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PREFACE

With this volume, the *Annual Review of Biochemistry* enters upon its second decade. Let us look briefly into the events of this ten-year period.

The year 1932 was one of severe economic depression, but it was also a time of great scientific activity: research in many fields was rapidly bearing fruit. Many of us can recall the striking advances in steroid chemistry that were announced during the first few years of the decade—advances that made possible a resolution of the structure of many of the steroid hormones. The decade was rich in contributions to the chemistry of the vitamins. Much was added to our knowledge of proteins and of enzymes. Equally important was the progress made in the study of isolated reactions and integrated systems—hydrogen transfer, phosphate transfer, carbon-dioxide transfer, etc.

Progress in the political and social sciences, in the machinery of government, and in the creation of adequate social controls against economic royalism and war was, however, disappointingly slow. After several years of grim foreboding, punctuated by acts of national aggression that passed almost unheeded, war broke out in 1939. Since then it has spread over the entire continent of Europe and has virtually engulfed the world. It is a sad commentary on the instincts and mind of man that he has not yet succeeded in building up a rational social order and in applying the endless discoveries in the natural and physical sciences solely to good ends.

Intellectual cooperation with the peoples of Europe and of much of Asia has become almost impossible. The darkness of the Middle Ages has descended over half of the earth. Libraries have disappeared, the pursuit of learning has become increasingly difficult, science has been turned to unhappy ends, scientific publications appear irregularly if at all, and the exchange of scientific periodicals between the two halves of the earth is almost at an end.

Because of these considerations it is a remarkable, if not unexpected, fact that there has been little diminution in the scientific productivity of the free peoples of the earth. *Chemical Abstracts* has observed no appreciable decrease in the number of papers to be abstracted. The scientific periodicals of Great Britain are not far short of peacetime size. American journals have tended to increase in size

and in number. There has been no significant diminution as yet in the number of papers reviewed in the *Annual Review of Biochemistry*—4,123 in Vol. IX (1940), 3,933 in Vol. X (1941), and 3,929 in the present volume.*

Mention should be made of the publication of a *Cumulative Author and Subject Index* which was released from the press in January of this year. Inasmuch as individual subject indexes had not been prepared previously for Volumes I to V, we trust that the cumulative index will correct this deficiency and will prove to be worth while.

Again we wish to extend the thanks of the readers and of this Committee to those who have prepared the present volume. Whatever merit the *Review* may possess is due entirely to their generous collaboration in authorship. Year after year this assistance has been gladly given, despite the increasing burdens assumed by one and all since the advent of war. We regret that circumstances beyond control prevented completion of the reviews by Professor Robinson, Professor Hammarsten, and Professor Wolfrom, announced in the fall prospectus. We must also record, and with great regret, the death of two of our present collaborators, Dr. A. H. K. Petrie, who passed away in Australia on January 10, 1942, at the early age of thirty-eight, and Dr. Elma Tufts Cohn, who collaborated with J. C. Aub and W. E. Cohn in preparing the review on "Clinical Aspects of Calcium and Phosphorus Metabolism."

Finally we express our appreciation of much editorial aid which was given by Professor Hubert Loring, of the splendid help that was rendered by our editorial assistants, and of the careful work of the Stanford University Press. We trust that typographical and related errors will be brought to our attention for notation on the errata sheet of Volume XII.

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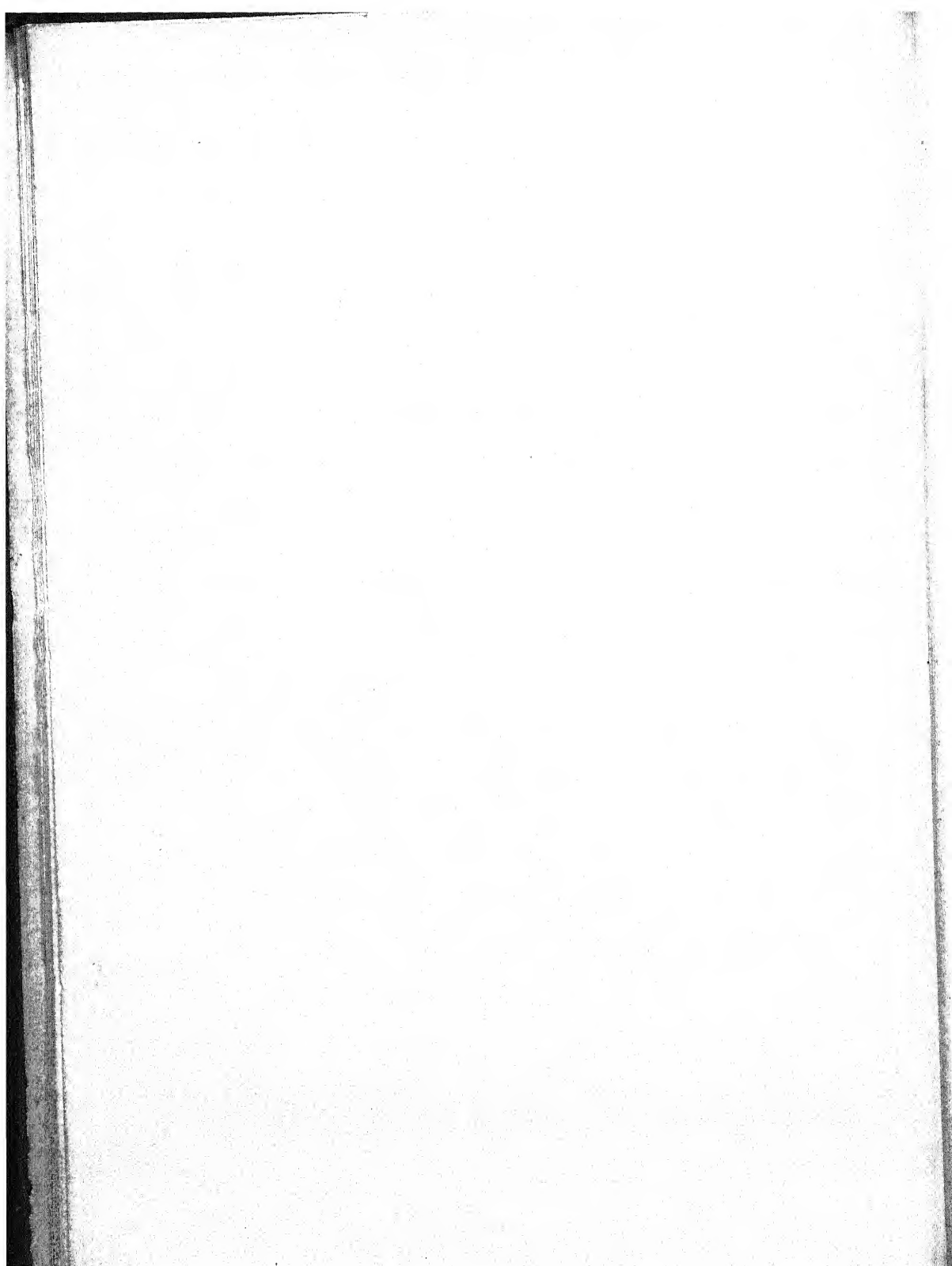
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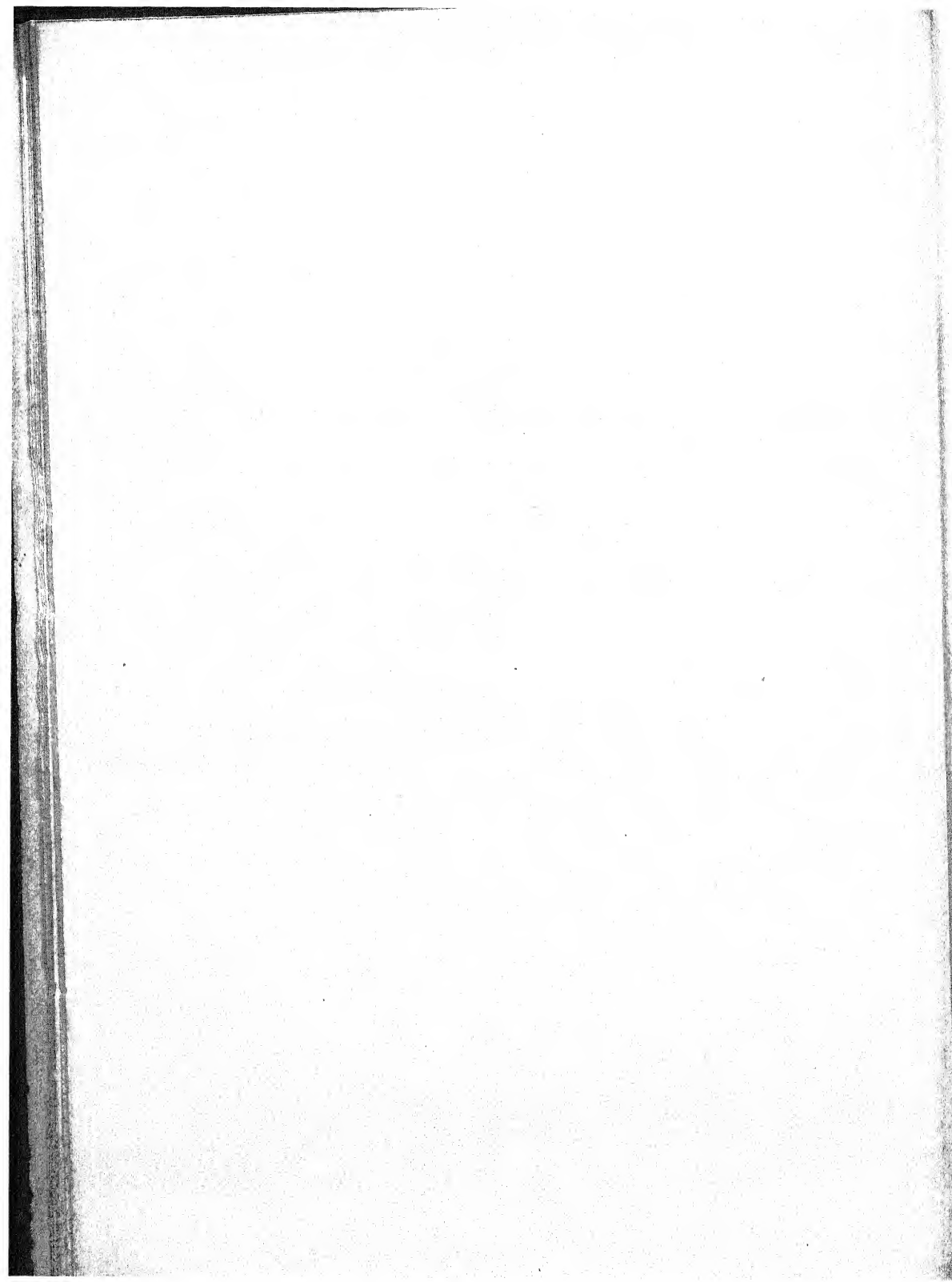
ERRATA

- Volume V, page 8, line 6: *for* phosphatase, *read* phosphatase.
Page 547, lines 6 and 7: *for* protocatechinic, *read* protocatechuic.
- Volume VI, page 115, line 9 from bottom: *for* protargon, *read* protagon.
- Volume VII, page 83, line 22: *for* Grandilla, *read* Granadilla.
- Volume VIII, page 202, line 2 from bottom: *for* lyotropic, *read* lipotropic.
Page 202, last line: *for* lyotropic, *read* lipotropic.
- Volume IX, page 479, line 22: *for* Tangeretin, *read* Tangeritin.
- Volume X, page 9, line 7: *for* +0.140v., *read* +0.186v.
Page 132, line 9: *for* to 1.62 per cent and of this sulfur 4.32 per cent was present as cystine, *read* to 1.62 per cent; 4.32 per cent of cystine was present.
Page 497, line 26: *for* (+) lactone, *read* (—) lactone.
Page 497, line 27: *for* (—) lactone, *read* (+) lactone.
Page 566, line 2 from bottom: *for* racemiase, *read* racemase.
Page 687, column 2, line 14: *for* racemiase, *read* racemase.



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BIOLOGICAL OXIDATIONS AND REDUCTIONS

By ERIC G. BALL

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No new salients have been described in this field within the last year. There has been, however, considerable activity in pushing forward those salients that have already been established in the past with the result that some important new ground has been gained. This review will be chiefly concerned with those advances made in the studies on the individual enzyme systems concerned in biological oxidations. These will be grouped for the purposes of discussion according to the nature of their prosthetic groups. Communications dealing with the larger aspects of tissue oxidations or the action of inhibitors on such oxidations will be largely omitted due to lack of space.

Iron proteids.—Among the iron-containing enzymes, cytochrome oxidase and the three cytochromes undoubtedly rank among the most important. With the exception of cytochrome-*c*, the study of the properties of the cytochromes has been handicapped by the fact that they resisted separation from each other. Therefore, the reports of Yakushiji & Okunuki (1) and of Straub (2) that they have isolated cytochrome-*a* from heart muscle constitutes a notable advance. The procedure employed by both laboratories appears to be similar. Sodium cholate is used to effect a clearing of the colloidal suspension of the cytochromes. After several intermediate manipulations, cytochrome-*a* is precipitated by the addition of ammonium sulfate. Straub finally obtains a solution which, he claims, contains both cytochrome-*a* and -*a*₃ and shows a strong Tyndall effect. This preparation oxidizes neither succinate nor *p*-phenylenediamine but will oxidize the latter if cytochrome-*c* is added. The reduced preparation shows two main absorption bands: the one, centered at 610 mμ, tends to be shifted slightly to shorter wave lengths in the presence of carbon monoxide; the other, located at 443 mμ, is shifted to 430 mμ with carbon monoxide. Keilin & Hartree (3), using a heart muscle suspension, assign a value of 605 mμ to the main band of the reduced form of cytochrome-*a* and -*a*₃ and report that the carbon monoxide compound of *a*₃ shows bands at 590 and 432 mμ. Melnick (4), in a preliminary report on the photochemical spectrum of cytochrome oxidase in heart muscle,

assigns the values 589, 510, and 450 $m\mu$ to the chief absorption bands of the carbon monoxide compound of this enzyme. Straub states that the oxidized preparation obtained by him shows a band centered at 420 $m\mu$ which is shifted to 430 $m\mu$ on the addition of cyanide. He concludes that the existence of cytochrome- a_3 and its identity with cytochrome oxidase is thus established. Before accepting this conclusion, it would be of interest to know if the oxidation of *p*-phenylenediamine by the reconstructed system is inhibited by carbon monoxide and if so, the effect of light on the inhibition. There seems to be no doubt, however, concerning the dual nature of what was formerly called cytochrome- a . Earlier, the writer (5) observed that the broad band of reduced cytochrome- a , centered at 605 $m\mu$, underwent an uneven fading as the oxidation-reduction potential of the medium was increased. A broad weak band centered at 595 $m\mu$ still remained visible after the main band had faded. It may well be that this band is now to be attributed to the reduced form of cytochrome- a_3 . In this case, it may be stated that the oxidation-reduction potential of cytochrome- a_3 is positive to that of the cytochrome- a system. This is a relationship which might be expected if cytochrome- a_3 is to be identified with cytochrome oxidase.

The advantages to be gained by the isolation of each of the cytochromes is well demonstrated by a series of four papers by Theorell & Åkesson (6) on the properties of cytochrome- c . These authors, using a preparation purified by electrophoresis, have carried out a series of studies on cytochrome- c which included amino acid analyses, spectrophotometric determinations, acid-base titration curves, and magnetic susceptibilities. On the basis of a sulfur content of 1.47 to 1.48 per cent, and assuming six atoms of sulfur per molecule, the molecular weight of cytochrome- c is calculated to be 13,120. The sulfur-containing amino acids remain, as yet, unidentified. The amino acid content of cytochrome- c appears to be unusual in two respects: first, its low content of histidine (3.3 per cent); and second, its high content of lysine (24.7 per cent). If each molecule contains ninety-six amino acids, twenty-two of these may be accounted for by lysine residues. Such a high content of this amino acid is in keeping with the alkaline isoelectric point, pH 10.65 (0°C.), of the compound. Details of the studies on the absorption spectra of ferricytochrome- c at different pH values, the acid-base titration curves of ferro- and ferricytochrome- c , and magnetic measurements of both forms cannot be given here. The results, however, permit the authors to draw further conclusions con-

cerning the linkage of the heme group to the protein component in cytochrome-*c*. It will be recalled that Theorell (7) earlier advanced the view that the protoporphyrin of cytochrome-*c* was linked to protein through a thio-ether linkage as a dicysteine adduct. This view he later rejected (8), but now he appears to accept it again in view of the findings of Zeile & Meyer (9). In addition to this linkage, the evidence now presented indicates that two of the total of three imidazole groups present in cytochrome-*c* constitute the hemochromogen forming groups. These two histidine imidazole groups appear to be strongly bound to the iron of the heme group at intermediate pH values. Thus all six valences of the iron in cytochrome-*c* are accounted for since four must be assigned to the porphyrin nitrogen atoms. The authors point out that this is in contrast to hemoglobin where only one imidazole group appears to be favorably placed for co-ordination with the iron atom. As a result, hemoglobin has one of the six octahedral valences of the iron atom left free for addition of oxygen, carbon monoxide, etc. Cytochrome-*c* with no free valences is thus not autoxidizable since oxygen is unable to approach the iron atom. Cyanide or carbon monoxide likewise cannot form compounds at physiological pH values. However, in strongly acid or alkaline solutions, one or both of the imidazole groups of cytochrome-*c* may be freed from the iron atom. Autoxidation of cytochrome-*c* or its combination with cyanide or carbon monoxide at these pH values is thus explained.

It is difficult to reconcile these conclusions with those of Potter (10). He finds that the incubation of cytochrome-*c* with cyanide prevents its reduction by succinate in the presence of a heart muscle preparation. The degree of inhibition depends on the length of time of incubation and the concentration of cyanide. Incubation of cyanide with the heart muscle preparation is without effect. From this evidence and a shift in the absorption spectrum of ferricytochrome-*c* on addition of cyanide, he concludes that cyanide combines with ferricytochrome-*c*.

Numerous studies have appeared on the occurrence of cytochrome oxidase and the cytochromes in various types of living tissue. Krahle *et al.* (11) have shown that though *Arbacia* eggs contain no cytochromes, it is possible on the addition of cytochrome-*c* to demonstrate the presence of a substance with properties similar to cytochrome oxidase. A similar situation may exist in wheat embryos according to Brown & Goddard (12). Whether this substance resembling cyto-

chrome oxidase is functionally active in these tissues without the presence of cytochromes remains to be answered. If it is, then we must broaden our definition of cytochrome oxidase before we apply this name to such substances.

Such studies as these raise the question of how the dehydrogenases are linked to oxygen even in those tissues which contain cytochrome. It appears to be definitely established that the reoxidation of the reduced pyridine nucleotides occurs by way of a flavoprotein. The substance in animal tissues responsible for the reoxidation of the reduced flavoprotein is, however, not known. Certainly its direct reaction with cytochrome-*c* does not seem to occur. Lockhart & Potter (13) present evidence that a substance intermediate to cytochrome-*c* and flavoprotein is needed and favor earlier suggestions that cytochrome-*b* may be involved. Michaelis & Quastel (14) report experiments which they believe suggest that narcotics block respiration by acting on some substance linking flavoprotein and cytochrome oxidase. Since they find that narcotics do not inhibit the oxidation of *p*-phenylenediamine nor of succinate, it would appear that its site of action must lie at least below cytochrome-*c* in the chain. A somewhat similar situation is pictured by Greville (15) for the action of narcotics on tumor tissue. According to this worker, the site of action of dinitro-*o*-cresol in stimulating respiration in tumor tissue lies at or above the link in the chain blocked by narcotics. Valuable as such studies are, it is possible that an answer to the question of the normal pathway in animal tissue will have to await the further separation of the components of the cytochrome system. Indeed, even the accomplishment of this feat may not tell the whole story, for Stannard (16) now has presented further evidence for his hypothesis, based on the action of inhibitors, that different mechanisms of respiration exist in resting and active muscle. The elucidation of the oxidative pathways in various lower organisms may also furnish important clues. However, it must always be borne in mind that oxidative mechanisms in different organisms may differ greatly. Further examples of this fact appear in the work of Keilin & Harpley (17) on the cytochrome system of *Escherichia coli commune* and in a similar study by Baker & Baumberger (18) on a ciliate protozoan. Stier & Castor (19) have even developed a substrain of yeast that contains no cytochrome oxidase by culturing in a medium containing cyanide.

Schultze (20), continuing his work on the relation of copper to cytochrome oxidase, has shown that the cytochrome oxidase activity

of bone marrow of rats is greatly decreased on a copper-deficient diet. Administration of copper restored the cytochrome oxidase content of the marrow to normal within twenty-four hours. A rapid increase of the cytochrome oxidase activity of bone marrow was also observed in response to such hematopoietic stimuli as hemorrhage, low oxygen tension, or feeding of cobalt. Schultze concludes that a close relation exists between cytochrome oxidase activity of bone marrow and its ability to form hemoglobin and erythrocytes.

Since succinic oxidase activity is so closely bound up with the cytochromes, its discussion under this heading is perhaps not amiss. One of the most interesting findings concerning this enzyme is that reported by Axelrod, Swingle & Elvehjem (21) that calcium has a marked stimulatory effect upon its activity. Using homogenized suspensions of liver, kidney, or heart as a source of the enzyme, a maximum effect was obtained upon addition of 20 μ g. of calcium to 20 mg. of tissue. The greatest response was obtained with heart tissue, increases in succinic oxidase activity as great as 200 per cent being encountered. One cannot help wondering what relationship this effect of calcium bears to the well-known effects of calcium ion on heart muscle contractions or to the well-known inhibitory effect of fluoride upon the activity of this enzyme system. Borei (22) has recently concluded that fluoride poisons the succinoxidase system by acting on some substance intermediate to cytochrome-*c* and succinic dehydrogenase. It is not known as yet at what point in the succinic oxidase chain calcium exerts its influence. Potter (23) in a study of similar tissue homogenates reached the conclusion that cytochrome-*c* was the only limiting soluble factor in the activity of succinoxidase. A marked drop in the succinic dehydrogenase activity of heart and skeletal muscle of guinea pigs on a diet deficient in ascorbic acid was reported by Harrer & King (24).

Few studies on catalase have appeared. Michaelis & Granick (25) reported that the magnetic moment of catalase is 4.6 ± 0.3 , a value nearly the same as that found for ferric hemoglobin hydroxide. The catalase content of liver has been shown to be lowered on a copper-deficient diet (26), or in rats carrying subcutaneously implanted hepatic tumors (27). Removal of the tumors restored the liver catalase content to normal within forty-eight hours.

Two reports on peroxidase deserve mention. In a report available to the writer only in abstract, Theorell (28) has reported the separation of two peroxidases from horse-radish by electrophoresis at pH

7.5. The peroxidase which migrates to the cathode at this pH will oxidize dihydroxymaleic acid directly and is inhibited by $0.00001\ M$ hydrogen cyanide. The other migrates to the anode at pH 7.5 and will oxidize dihydroxymaleic acid only after the addition of hydroquinone. It is insensitive to hydrogen cyanide. The prosthetic group of this peroxidase is split off by treatment with acidified acetone at -15°C . The colorless protein fraction so obtained can be centrifuged off and redissolved in 1 per cent sodium bicarbonate solution. It shows only 0.6 per cent of the activity of the original preparation. If blood hemin is now added, the activity is restored to 93 per cent of the original value.

Lipmann (29) reports that peroxidase obtained from horse-radish catalyzes the oxidation of *p*-aminobenzoic acid by hydrogen peroxide. A red oxidation product is produced. The reaction is inhibited by sulfanilamide at a concentration of 33×10^{-8} moles per liter. Sulfathiazole and sulfapyridine also inhibit the reaction.

Stern & Melnick (30) have published the detailed account of their study of what they term the Pasteur enzyme of retina tissue. Since the considerations leading up to this study and the main findings have been presented by Stern and Barron in the two preceding numbers of the *Annual Review of Biochemistry*, there is no need to repeat them here. Stern & Melnick now form the following picture of the action of this enzyme:

The Pasteur enzyme contains iron which during the catalysis may exist alternately in the ferric and the ferrous forms. The *ferrous* form is autoxidizable; i.e., it is capable of reacting spontaneously with molecular oxygen to yield the ferric form, possibly via an oxygenated, short-lived intermediate analogous to oxyhemoglobin. The *ferric* form inhibits fermentation, probably by maintaining a component of the glycolytic system in the oxidized, inactive state.

Now, such a mechanism implies that oxygen is continually being used up to maintain the component of the glycolytic system in the oxidized state. When, however, the reaction of oxygen with the Pasteur enzyme in retina is blocked by carbon monoxide, there is no apparent decrease in oxygen consumption of retina tissue as might be expected on this basis. Stern & Melnick dismiss this difficulty by saying that it is possible that the absolute amount of molecular oxygen required for this purpose is very small, owing to a slow or negligible rate of reduction of the component of the glycolytic system which reacts with the Pasteur enzyme. If this is the case, then the establishment of a maximum glycolysis rate after poisoning the Pasteur enzyme

with carbon monoxide should be a relatively slow process. No data seem to be available to determine whether this is the case.

Stern & Melnick (30) compared the photochemical absorption spectrum of their Pasteur enzyme in retina with the corresponding spectrum of cytochrome oxidase in yeast, in order to show that the two enzymes are different. A far more satisfying procedure is the comparison of the photochemical spectra of the two enzymes as they occur in one and the same tissue. Melnick (31) has performed such a study for baker's yeast. In yeast, unlike retina, there is a simultaneous decrease in respiration as glycolysis increases in the presence of carbon monoxide. Melnick has, therefore, determined the effect of light of known wave length in altering the effect of carbon monoxide on both respiration and glycolysis. When the absorption spectra so obtained are plotted, it is found that they are nearly identical. The positions of the three main bands coincide exactly whether the increase in oxygen uptake or the decrease in carbon dioxide production is used as an index. One difference only exists and that is that light in the neighborhood of 560 m μ appears to be more effective in dissociation of the carbon monoxide complex if oxygen uptake rather than carbon dioxide production is the index used. Melnick concludes on the basis of this single difference that two different compounds are involved: cytochrome oxidase controlling respiration, and the Pasteur enzyme controlling glycolysis. The data might be interpreted as an intrinsic coupling of respiration with fermentation. The marked similarity of the absorption spectra, regardless of whether glycolysis or respiration is used as an index, might be interpreted to indicate that one and the same substance is controlling both processes. The difference in action of light at 560 m μ on the two processes might just as readily be interpreted to indicate that an additional although minor carbon monoxide sensitive factor enters into respiration processes but is not connected with the main respiratory pathway or the inhibition of fermentation.

Other ingenious explanations of the Pasteur mechanism have also been published recently. Johnson (32) and Lynen (33) emphasize the role of phosphorylation processes in the mechanism. Johnson, for example, points out without giving any supporting data that oxidative phosphorylation processes might be able to proceed at inorganic phosphate and phosphate acceptor concentrations which are much lower than those required to carry on glycolytic phosphorylation mechanisms. Gottschalk (34) prefers to attribute the Pasteur effect to the

more rapid oxidation of reduced diphosphopyridine nucleotide by acetaldehyde than by the cytochrome system. He believes that the slower rate of utilization of sugar by yeast under aerobic conditions is, therefore, due to the fact that acetaldehyde is removed as it is formed and the reduced coenzyme must perforce be reoxidized by the slower cytochrome route. This appears to be another way of saying what has been proposed before.

Copper proteids.—Oxidative enzymes that contain copper may be divided into two groups according to their substrates. One group would thus contain those enzymes which oxidize mono- or polyphenols; the other, those enzymes whose substrate is ascorbic acid. Enzymes in one group do not appear to be able to catalyze the oxidation of the substrate for the other group. However, enzymes of both groups presumably catalyze the oxidation of their substrates by reason of the change of copper from the cupric to the cuprous form, the latter being reoxidized by oxygen. Though the oxidation-reduction potentials of these copper systems are not known, it seems unlikely that a difference in the potential of the enzymes belonging to each group can account for their substrate specificity. If this were the case, then those enzymes capable of catalyzing the oxidation of catechol, etc., should also be able to catalyze the oxidation of ascorbic acid, since the ascorbic acid system has an oxidation-reduction potential quite negative to that of the catechol system.

The study of ascorbic acid oxidase activity is complicated by the fact that ionic copper is able to catalyze the oxidation of ascorbic acid by air. This fact has received careful consideration in the studies of Meiklejohn & Stewart (35). Using cucumber juice as the source of the enzyme, these authors claim that the ascorbic acid oxidase activity of the juice is proportional to the amount of copper in the juice after the "free" copper has been removed by dialysis. The juice can be completely inactivated if it is dialyzed against sodium cyanide according to the method of Kubowitz for removing bound copper. Activity is restored to such a preparation by the addition of copper. This activity is far in excess of that exerted by a corresponding amount of copper alone.

Crook (36) has presented evidence to show that the enzyme responsible for catalyzing the reduction of dehydroascorbic acid by SH-glutathione is not identical with ascorbic acid oxidase.

The processes of enzymic oxidation of the mono- and polyphenols though not affected by ionic copper are complicated by the fact that

during oxidation of the substrate the enzyme may be adversely affected. Different workers have, therefore, adopted different procedures to overcome this difficulty. As a result, an intercomparison of studies from different laboratories on these enzymes is often impossible. Investigators often change their methods completely as their knowledge increases, as is witnessed by the recent studies reported by Miller & Dawson (37). Further complications are introduced because purification of an enzyme often causes it to lose its action upon one of its substrates. For example, Venkateswaran & Sreenivasaya (38) describe the preparation of an enzyme from *Dolichos lablab* whose activity towards phenol diminished as purification progressed, though its activity towards catechol remained unchanged. To account for this, the authors suggest that a factor, which is in part replaceable by catechol, is eliminated in the purification process.

The occurrence of enzymes capable of oxidizing mono- and polyphenols has been described recently in a variety of materials. Charles & Rawles (39) report that extracts of black or red chicken feather germs show a cyanide-sensitive, heat-labile tyrosinase activity that is not exhibited by white feathers. Smirnov & Pshennova (40) find that tobacco contains an enzyme that readily catalyzes the oxidation of hydroquinone. Other polyphenols or monophenols inhibit this action. The possible importance of a polyphenol oxidase in the fermentation reaction of tea leaves is pointed out by Lamb & Sreerangachar (41). The occurrence in the ink sac of the squid, *Loligo pealii*, of an enzyme capable of catalyzing the oxidation of dihydroxyphenylalanine is described by Ball & Ramsdell (42). Its action is inhibited by the usual reagents which form copper complexes.

The importance of copper for protyrosinase has been demonstrated by Allen & Bodine (43). Protyrosinase is a substance obtained by these authors from grasshopper eggs which on treatment with suitable agents yields active tyrosinase. Dialysis of protyrosinase against cyanide solutions causes protyrosinase to lose four fifths of its copper and most of its potential activity. If copper is added to such a dialyzed preparation, it will again yield tyrosinase upon addition of the activating agent.

The possibility that copper alone is not the only prosthetic group required for the action of enzymes capable of acting on polyphenols is raised by the observations of Keilin & Mann (44) on laccase from the latex of the lacquer tree. These workers were able to show that the laccase obtained from three different sources was blue in color and

contained both copper and a blue pigment. Attempts to obtain an enzyme free from this blue pigment were unsuccessful. The blue pigment, however, could be obtained free from both copper and protein. It could be reversibly reduced to a leuco compound which suggests that it may play a role in the oxidative mechanisms catalyzed by the enzyme.

Several papers have appeared reporting studies on factors that affect the activity of tyrosinase. Wisansky, Martin & Ansbacher (45) state that a tyrosinase preparation obtained from mushrooms is inhibited by *p*-aminobenzoic acid when tyrosine or dihydroxyphenylalanine are substrates. However, the acid has an accelerating effect on the enzyme's action if *p*-cresol is the substrate. What relationship this action of *p*-aminobenzoic acid bears to its vitamin action remains to be determined. Figge (46) claims that melanin formation due to tyrosinase action can be regulated by the oxidation-reduction potential of the medium. Optimum conditions for melanin formation are claimed to exist between the potential range $+0.3$ v. and -0.3 v. Using potato tyrosinase, Figge & Allen (47) report that addition of estrone speeds up the release of the inhibition of melanin formation that is produced by glutathione. They discuss the possibility that tyrosinase attacks estrone itself to form a quinone which can, in turn, bring about the oxidation of glutathione and thus release tyrosinase from its inhibiting effect. Westerfeld (48) reported earlier that tyrosinase is capable of inactivating estrone.

Flavoproteins.—Only one new flavoprotein has been added to the list this year. This one was obtained by Green, Knox & Stumpf (49) from top brewer's yeast. Its function is unknown since it was inactive towards all substrates tried. It resembles xanthine oxidase in possessing a brownish red color and in containing flavin adenine dinucleotide as a prosthetic group. The purest preparation obtained contained 0.86 per cent flavin phosphate. This preparation, however, in the ultracentrifuge appeared to be only 60 per cent pure.

The question as to whether in animal tissues the reduced pyridine nucleotides require separate pathways for their oxidation is still unsettled. Abraham & Adler (50) have studied the ability of various tissue extracts to catalyze the reoxidation of the two reduced pyridine nucleotides. They find that heart extracts are 130 times more active in reoxidation of reduced diphosphopyridine nucleotide than triphosphopyridine nucleotide. In adrenal gland extracts, the ratio of these activities is 4.5, and this ratio could be altered by precipitation of the

extract at pH 4.5. The authors conclude that the two reduced pyridine nucleotides require different enzymes for their reoxidation. They offer no proof that these enzymes are flavoproteins.

The relation of experimental riboflavin deficiency to flavoprotein enzymes has been further studied by Axelrod & Elvehjem (51). They find that the xanthine oxidase content of rat liver is markedly decreased on a riboflavin deficient diet. A diminution of the protein part of the enzyme seems to have occurred since the addition of the prosthetic group of xanthine oxidase to the liver homogenates from the deficient animals did not restore activity. However, the administration of riboflavin to the diet of the deficient animals caused a rapid restoration of the liver xanthine oxidase to normal values. Greenstein *et al.* (52) have also shown that the xanthine oxidase content of cancerous hepatic tissue of the rat is lower than that of normal hepatic tissue.

The association of flavin with a purified virus has been reported by Hoagland *et al.* (53). The elementary bodies of vaccinia have yielded a flavin which, as shown by its ability to function as a co-enzyme for *D*-amino acid oxidase, appears to be flavin adenine dinucleotide. The concentration of riboflavin is reported to be of the order of 1.1 to 1.5 mg. per 100 gm. of virus.

The specificity of the *D*-amino acid oxidase continues to evoke interest since workers fail to agree on this subject. Both Klein & Handler (54) and Karrer & Frank (55) have recently published papers dealing with this subject. Their results do not agree. Both laboratories have worked at pH 8.3 so this factor, which incidentally has not been carefully considered in earlier comparisons of results, cannot explain the difference in results. Most workers appear to agree that *D*-glutamic acid and *D*-lysine are not attacked by the isolated system. It would be of interest to know whether oxidation of these amino acids would occur at pH values nearer to their isoelectric points than have been heretofore employed in such studies. Handler, Bernheim & Klein (56) find that the *N*-methyl derivatives of *DL*-methionine, *DL*-alanine, and *DL*-leucine are oxidized by *D*-amino acid oxidase preparations as well as by broken cell preparations of rat kidney and liver. The liver preparation, in addition, has the ability to oxidize *DL-N*-methylhistidine.

Birkofer & Wetzel (57) confirm earlier reports that kidney contains more *D*-amino acid oxidase than liver and report that herbivorous animals have in general a lower content of this enzyme in their

tissues. The higher content of *d*-amino acid oxidase in kidney than in liver is of interest in connection with the findings of Bliss (58) that the *d*-forms of amino acids are a better source of urinary ammonia than the *l*-forms; while MacKay *et al.* (59) report that the *d*-form of alanine is inferior to the *l*-form as a precursor of liver glycogen.

The inhibition of *d*-amino acid oxidase by benzoic acid is reported by Klein & Kamin (60).

Pyridine nucleotides.—As far as the writer is aware, no new enzymes have been described that require one of the pyridine nucleotides as coenzyme. Most of the studies dealing with these compounds that have been reported are concerned with either their isolation or their quantitative determination in various tissues. Williamson & Green (61) describe an abbreviated method using previously described techniques for obtaining diphosphopyridine nucleotide (DPN)¹ samples of about 60 per cent purity. Jandorf (62) introduces the use of charcoal as an adsorbent in the purification of the compound. However, the task of obtaining a sample of known purity still remains an arduous one.

Determination of the DPN content of tissues is receiving more and more attention. The methods available for such determinations appear to be accurate enough. Schlenk & Vowles (63) have described further improvements in the yeast fermentation test. Jandorf, Klemperer & Hastings (64) have published a new procedure using muscle enzymes which have been freed from their normal content of DPN by charcoal treatment. The chief difficulties that lie in the path of the investigator who desires to make such determinations appear to be as follows: (*a*) the attainment of a sample of DPN of known purity to use as a standard in the enzyme tests; and (*b*) the prevention of destruction of the DPN of the tissue during the preparation of the sample for analysis. The discordant values given by various investigators for the normal DPN content of tissues suggest that perhaps these difficulties have not been fully recognized by all workers. The establishment of a standardized preparation of DPN as a reference for all work would help overcome the first difficulty mentioned. The destruction of DPN by tissue extracts has been investigated by several workers. Lennerstrand (65) has presented evidence to show that a gradual destruction of DPN occurs even in the yeast fermentation test used to measure DPN. He finds the rate of destruction is much

¹ In the following paragraphs this nucleotide will be referred to as DPN.

greater, however, if the fermentation reaction is blocked by fluoride. Indications are presented that the active ferment system is capable not only of preventing the destruction of DPN but that it may also be capable of effecting a synthesis of DPN from its split products. Mann & Quastel (66), using the lactic acid dehydrogenase system as a measure of DPN activity, note that brain suspensions rapidly destroy DPN and postulate that the action is due to a nucleotidase. Rat liver and kidney extract contain a similar destructive agent, though skeletal and heart muscle are free from it. Mann & Quastel claim that the addition of nicotinamide prevents the destructive action. Lennerstrand, using yeast preparations, observed no protective action of nicotinamide.

Handler & Dann (67) report that a gram of normal rat liver contains about 400 μg . of both pyridine nucleotides. Katzenelbogen, Axelrod & Elvehjem (68), on the other hand, find that a gram of normal rat liver contains 1000 μg . of DPN alone. The latter also report that the content of DPN in liver fell markedly in rats with experimental hyperthyroidism which were fed rations low in nicotinic acid. Control animals on similar diets showed no such change.

The widespread occurrence of the pyridine nucleotides in different organisms continues to be demonstrated. Still (69) has shown that *Escherichia coli* possesses a triosephosphate dehydrogenase system similar to that of yeast. Hutchens, Jandorf & Hastings (70) report that *Chilomonas paramecium* is able to synthesize DPN in a solution containing ammonia as the only nitrogen source and acetate as the sole source of carbon. Saunders, Dorfman & Koser (71) present evidence which they believe indicates that nicotinamide is concerned in cellular oxidations of dysentery bacilli in some way other than in the formation of the pyridine nucleotides.

Altschul, Persky & Hogness (72) have recently published a short note describing a baker's yeast preparation that catalyzes the reaction between cytochrome-*c* and reduced DPN. The nature of the enzyme is not stated. Haas (73) has earlier mentioned the fact that preparations obtained from brewer's yeast could catalyze the reaction of reduced DPN with cytochrome-*c* or with methylene blue. On further purification of such preparations, Haas isolated a flavoprotein that catalyzed only the reaction of reduced DPN with methylene blue. Whether factors in addition to this flavoprotein or factors entirely independent of it were responsible for the cytochrome-*c* reaction of the crude preparation was not determined.

Diphosphothiamin, pyruvate metabolism, and carbon dioxide fixation.—The isolation of intact carboxylase from yeast has been undertaken by two different laboratories. Kubowitz & Lüttgens (74) in a preliminary report state that the enzyme contains 1 gram molecule of diphosphothiamin and 1 gram atom of magnesium per molecule of protein. The protein is assigned a molecular weight of 75,000. Green, Herbert & Subrahmanyam (75) in a more detailed report state that their best preparation contained 0.46 per cent of diphosphothiamin and 0.13 per cent magnesium. The authors state that this preparation is not homogenous. Conversion of these values into the terms used by Kubowitz & Lüttgens would mean that each molecule of enzyme contained about 4 to 5 gram atoms of magnesium and one gram molecule of diphosphothiamin. Both laboratories appear to agree that dissociation of the enzyme into its component parts occurs best at pH values in the vicinity of 8, and that dissociation does not occur to any appreciable extent at pH 5 to 6. According to Green *et al.*, the recombination of the three components (magnesium, diphosphothiamin, and protein) to form the active enzyme is a complex affair. It is possible that the use by these authors of citrate buffer as a medium for such recombination tests has not furnished the optimum conditions for the participation of magnesium ion.

The role of thiamin in reactions other than the mere decarboxylation of pyruvate is receiving more and more attention. Still (76) has shown that cell-free extracts of *Escherichia coli* oxidize pyruvic acid to acetic acid and carbon dioxide. The enzyme system is comprised of protein or proteins, inorganic phosphate, diphosphothiamin, and magnesium or manganese. Flavin adenine dinucleotide may also be a component. Quastel & Webley (77) claim that the oxidation of acetic acid by propionic bacteria requires thiamin. In animal tissues, Barron *et al.* (78) present evidence which indicates that diphosphothiamin is involved in the metabolism of α -ketoglutarate.

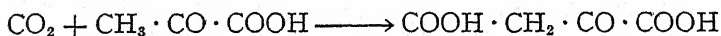
In the last few years, the study of the breakdown of pyruvic acid to smaller units has been overshadowed by studies that show that it may also undergo reactions leading to the formation of compounds containing more than three carbon atoms. One such reaction is the formation of acetylmethylcarbinol according to the following equation:



Silverman & Werkman (79) state that a cell-free enzyme preparation can be obtained from *Aerobacter aerogenes* that catalyzes this

reaction. The presence of a similar enzyme system in animal tissues is reported by Green *et al.* (80). Both the animal and bacterial enzyme systems appear to require diphosphothiamin and either magnesium or manganese for their action. The animal enzyme system apparently differs from the bacterial in that aldehyde appears to be an intermediate in the reaction. Green *et al.* believe that pyruvate is first decarboxylated to acetaldehyde and that this product then condenses with pyruvate to yield an intermediate which in turn is decarboxylated to give acetylmethylcarbinol. In support of this view they present the following evidence: (a) in the presence of acetaldehyde, the rate of carbon dioxide formation from pyruvate is increased nearly four-fold; (b) from a given amount of pyruvate, the yield of acetylmethylcarbinol in the presence of acetaldehyde is twice that obtained with pyruvate alone; (c) when propionaldehyde is substituted for acetaldehyde in the system, the 2,4-dinitrophenylosazone of propionylmethylcarbinol can be isolated from the reaction mixture.

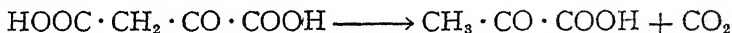
The formation of α -ketoglutarate from pyruvate and carbon dioxide is another reaction involving the synthesis of pyruvate into a larger molecule. This reaction was first conclusively demonstrated by Evans & Slotin (81) using liver slices and radioactive bicarbonate. The exact mechanism of this reaction is unknown, but it has been suggested, though not proven, by several workers (81, 82, 83) that the first step in the process is the formation of oxalacetate according to the following reaction:



Wood *et al.* (83) have recently even proposed a scheme to explain the entire mechanism. Their scheme accounts for the fact that all of the carbon coming from carbon dioxide that appears in α -ketoglutarate is located in the carboxyl group in the α -position to the carbonyl group. This fact has been established by the use of carbon dioxide containing isotopic carbon not only by these workers but also by Evans & Slotin (84). Both these groups of investigators point out that this fact rules out the possibility that a symmetrical molecule such as citrate is the precursor of α -ketoglutarate in this reaction. Stare *et al.* (85) have recently presented evidence which leads them to reject citrate as an essential stage in the utilization of pyruvate. [The reader should consult Evans (86) or Elliott (87) for recent reviews of the citric acid cycle.]

The condensation of a three-carbon compound and carbon dioxide

was first proposed by Wood & Werkman (88) to explain the carbon dioxide assimilation and formation of succinate from glycerol by propionic bacteria. Wood *et al.* (89), using carbon dioxide labeled with heavy carbon, showed that the fixed carbon dioxide appeared in the succinate and exclusively in the carboxyl groups of this acid. They proposed that this reaction involved the breakdown of glycerol to pyruvate and its subsequent condensation with carbon dioxide to form oxalacetate which was in turn reduced to succinate. The ability of propionic bacteria to form pyruvic acid from glycerol had been demonstrated by Wood, Stone & Werkman (90). Krebs & Eggleston (91) have now demonstrated that propionic acid bacteria contain the necessary enzyme systems to convert oxalacetate to succinate. There thus remains only the direct proof that pyruvate and carbon dioxide do condense to yield oxalacetate to establish conclusively that the mechanism suggested by Wood *et al.* (89) is the correct one. As a step in this direction, Krampitz & Werkman (92) have reported a study on an enzyme from *Micrococcus lysodeikticus* which catalyzes the reverse reaction:



They find that magnesium ions are required while diphosphothiamin or thiamin are not essential for the reaction. They discuss the possibility of a reversal of this process if energy is supplied by a coupled reaction.

During the year other evidence that carbon dioxide fixation may be an integral part of intermediary carbohydrate metabolism has accumulated. Searle & Reiner (93) find that the utilization of glucose under anaerobic conditions by *Trypanosoma lewisi* is activated by carbon dioxide which is consumed in the reaction. The amount of assimilated carbon dioxide is equivalent to the amount of succinic acid formed. For reasons presented in their paper, these authors believe that oxalacetate is not an intermediate and favor the formation of glucose carbonate to explain their findings. Solomon *et al.* (94) report that the administration to rats of lactate and bicarbonate containing radioactive carbon results in the formation of liver glycogen which contains radioactive carbon. These authors postulate a pathway involving the condensation of pyruvate and carbon dioxide to form oxalacetate to explain their findings.

Evidence that thiamin is concerned in the synthesis of oxalacetate from pyruvate by *Staphylococcus* has been presented by Smyth (95).

In tissues obtained from avitaminotic animals, Barron *et al.* (96) have found that the addition of thiamin accelerates condensation reactions of pyruvate leading to the synthesis of carbohydrates, α -ketoglutarate, citrate, acetoacetate, and succinate. Since the thiamin must be incubated with the tissues previous to addition of the substrate, these workers believe that phosphorylation of the thiamin occurs before it becomes functional.

The coupling of phosphorylation with oxidation of pyruvate in dialyzed dispersions of pigeon brain has been studied by Ochoa (97). He finds that it is not possible to demonstrate the phosphorylation of adenylic acid in such dispersion after the addition of the supplements necessary for the oxidation of pyruvate. This appears to be due to the presence of an active phosphatase in the preparation which breaks down the phosphorylated adenylic acid and thus prevents its accumulation. If, however, catalytic amounts of adenylic acid along with glucose or hexosemonophosphate are added, it is possible to demonstrate the formation of hexosediphosphate. In the early stages of the reaction, at least two atoms of phosphorus are esterified for each atom of oxygen consumed in oxidizing the pyruvate. Ochoa, therefore, suggests that there must be more than one point of esterification in the chain of reactions by which one hydrogen pair is transported from pyruvate to the ultimate acceptor, oxygen. The writer is inclined to favor this suggestion, but it should be pointed out that such an interpretation is based upon the premise that only enough energy is released at each step in this chain to esterify but one phosphorus atom. Until more is known about such energy relations, there must be considered the possibility that enough energy might be released during the single transfer of a hydrogen pair or its equivalence in electrons to bring about the simultaneous esterification of two phosphorus atoms.

In a similar study, Colowick, Kalckar & Cori (98) have found that for each molecule of glucose oxidized in a dialyzed heart or kidney extract, five or six molecules of glucose are esterified. Here then not more than one atom of phosphorus is esterified for each atom of oxygen consumed.

In connection with phosphorylation mechanisms, the report of Ohlmeyer (99) is of interest. He employs a dialyzed maceration juice of baker's yeast to which diphosphopyridine nucleotide, inorganic phosphate, and magnesium have been added. This preparation will ferment hexosediphosphate, and if glucose is also present, it will be simultaneously phosphorylated to hexosemonophosphate. If glucose

or hexosemonophosphate and only a trace of hexosediphosphate are added, no fermentation occurs since the conversion of hexosemonophosphate to hexosediphosphate can apparently not take place. However, if one now adds a coferment that can be obtained from yeast *Kochsaft*, fermentation proceeds. The function of this new coferment is thus apparently the phosphorylation of hexosemonophosphate to hexosediphosphate. The new coferment resembles the known adenine nucleotides in properties but cannot be replaced by them in this reaction.

Amine oxidation and transamination.—The exact nature of the enzymes responsible for the oxidation of various amine compounds still remains unsolved. Zeller, Stern & Wenk (100) have presented evidence which suggests that a flavin may be concerned in the diamine oxidase reaction. The evidence, however, is fragmentary as yet.

Amine oxidases from different sources appear to differ markedly in their properties. Blaschko (101) has studied the amine oxidase in various tissues of the marine organism, *Sepia officinalis*. Unlike the mammalian enzyme, the amine oxidase of *Sepia* shows a marked preference for tyramine as a substrate. Borghi & Tarantino (102) report that the histidase of skin, in contrast to that of liver, requires oxygen for its action.

Lutwak-Mann (103) has investigated the enzymic decomposition of amino sugars. The enzyme is found in yeast, in bacteria, and in animals. In animals it occurs largely in the kidney cortex, testes, and brain. There is none in liver or muscle. The activity of the enzyme depends greatly on the integrity of cell structure. Using glucosamine, N-acetylglucosamine, and chondrosamine as substrates, this worker finds that there is a consumption of oxygen with the liberation of ammonia and the formation of an acid. No carbon dioxide is produced. Besides ammonia, the products formed have not been identified, but they can undergo further oxidation in the presence of diphosphopyridine nucleotide and muscle dehydrogenases.

The specific role which transamination reactions play in intermediary metabolism still remains obscure. Cohen & Hekhuis (104) find that the only transamination reaction that proceeds at appreciable rates in normal tissues is that between *l*-glutamate and oxalacetate. Pyruvate is a poor substitute for oxalacetate. Zorn (105) likewise does not confirm the findings of Braunstein & Kritzmann (106). Cohen & Hekhuis (107) find that transamination activities in tumors, fetal tissue, and regenerating liver are even lower than in normal tis-

sues. They suggest that an inverse relationship exists between transaminase activity and protein synthesis.

On the other hand, Braunstein & Bychkov (108) conclude that the oxidative deamination of mono carboxylic *l*-amino acids is in all probability an indirect one involving transamination with α -ketodicarboxylic acids and the subsequent deamination of the dicarboxylic amino acids. They claim to have effected the oxidative deamination of *l*(+)-alanine with the formation of ammonia under aerobic conditions in a cell-free solution. Glutamic acid transaminase and dehydrogenase, diphosphopyridine nucleotide, α -ketoglutaric acid and an autoxidizable hydrogen carrier such as methylene blue are needed.

Karrer, Koenig & Legler (109) find that liver brei consumes an additional amount of oxygen when octopine is added. Since *d*-amino acid oxidase does not oxidize this arginine derivative, they attribute this to an oxidation of the compound by the *l*-amino acid oxidase of liver. Except for the report of Braunstein & Bychkov mentioned above, the writer was of the opinion that the oxidation of *l*-amino acids had only been observed in tissue slices. Karrer *et al.* speculate upon the possible role of octopine in transamination reactions.

Evidence that the amino group of the dicarboxylic amino acids plays another role besides that demonstrated for transamination reactions is presented by Borsook & Dubnoff (110). These workers find that the addition to kidney slices of either aspartic or glutamic acid together with citrulline leads to the formation of arginine. An oxidative reaction thus occurs in which the amino group of the dicarboxylic acid furnishes an imino group for the formation of arginine from citrulline. The authors believe that a preliminary oxidation of the amino acids before they react with citrulline is ruled out as the mechanism of the reaction. Their reason is the fact that arsenite which does not inhibit the oxidation of the amino acids does, however, inhibit the formation of arginine. They postulate, therefore, the formation of an intermediate compound by the reaction of the amino group of the dicarboxylic acids with the keto group of citrulline. It is this intermediate compound which is believed to undergo oxidation and scission to yield arginine and the ketodicarboxylic acid. Evidence is presented which indicates that the oxidation may proceed under aerobic or anaerobic conditions. Proline, hydroxyproline, lysine, and ornithine may replace the dicarboxylic amino acids in this reaction. Evidence is adduced that they do so by being converted first to glutamic acid.

Unclassified oxidative enzymes and processes.—The enzyme christened "carotene oxidase" by Sumner & Dounce (111) has been rechristened "unsaturated fat oxidase" by Tauber (112) and "lipoxidase" by Süllmann (113). These changes in nomenclature are proposed since the oxidation of carotene appears to occur only indirectly as the result of enzymic oxidation of unsaturated fats or fatty acids. It is the oxidation product of these compounds which reacts with carotene. Though soybean has been the chief source of this enzyme, Strain (114) reports that the enzyme is also present in the seeds of various legumes. He claims that the enzyme catalyzes the oxidation only of those compounds containing a $-\text{CH}=\text{CH}(\text{CH}_2)_7\text{C}(\text{O})-$ group. He also finds that other substances besides carotene can undergo an induced oxidation. These include chlorophyll *a* and *b*, *p*-phenylenediamine, dihydroxyphenylalanine, and ascorbic acid. Süllmann (113) reports that the enzyme has no dialyzable component and that lecithin and α -tocopherol can act as inhibitors of the enzyme action when an unsaturated fat is the substrate. Lecithin is ineffective as an inhibitor if an unsaturated fatty acid is the substrate.

Glyoxalase has been found in cell-free extracts of *E. coli* by Still (115). He reports that cysteine as well as glutathione can act as coenzyme. Behrens (116) reports that isogluthathione and asparthione can act as coenzymes for yeast glyoxalase though neither is as effective as glutathione.

A cell-free solution of hydrogenase, the enzyme which activates molecular hydrogen, has been obtained by Bovarnick (117) from *E. coli communior*. Phelps & Wilson (118) report that this enzyme also occurs in *Azotobacter*. They find that fumarate and nitrate as well as methylene blue can act as acceptors of the activated hydrogen.

The uricase content of the livers of rats fed a zinc-deficient diet is normal according to Wachtel *et al.* (119). However, a marked rise in plasma uric acid occurs. These workers also report that uricase prepared from hog liver by the method of Davidson (120) shows an increase in zinc content as the purification proceeds.

Handler, Bernheim & Klein (121) report the oxidative demethylation of sarcosine to glycine by liver brei. They identify both glycine and formaldehyde as end products and postulate N-methylol as an intermediate.

Evidence continues to accumulate that different oxidative pathways exist in different organisms and even in different tissues from the same animal. Barron & Friedemann (122) stress this point in

their studies of oxidations by microorganisms which do not ferment glucose. According to these workers the inability of certain organisms to oxidize glucose is due to their inability to phosphorylate it, since the phosphorylated hexoses can be oxidized. All oxidations by these microorganisms appear to be inhibited by cyanide, including the oxidation of *d*-amino acids. It is perhaps fortunate that bacteria do differ in their oxidative mechanisms from those of animal tissues, or otherwise there might be but little hope of selectively killing with drugs those microorganisms that invade the animal body.

Oxidation-reduction potentials.—Several studies have appeared dealing with researches on the oxidation-reduction potentials of compounds of biological interest. Bowman (123) reports that the E'_0 of the chorionic gonadotropic hormone at pH 5.9 and 38° is +0.354 v. The shape of the titration curve suggests that one electron is involved. The biological activity of the hormone is decreased by oxidative changes and can be restored by treatment with strong reductants. Studies on the oxidation-reduction potentials of vitamin K_1 have been reported from two laboratories. Using the polarograph, McCawley & Gurchot (124) report an E_0 value of +0.328 v. Riegel, Smith & Schweitzer (125), employing the more conventional potentiometric method, report an E_0 value of +0.363 v. for vitamin K_1 when dissolved in 95 per cent ethanol which was 0.2 *N* in hydrochloric acid and lithium chloride.

Horowitz & Baumberger (126) now report that the pigment obtained from *Urechis* eggs has an E'_0 value at 25° and pH 7.0 of +0.186 v. Only one electron appears to be involved in the oxidation, and the potential of the system changes 0.06 v. per unit change in pH within the pH range studied, 5.3 to 10.0. The reduction of the pigment in an alkaline pyridine solution brings out the spectrum of a pyridine hemochromogen. The authors suggest that the pigment is related to the hemins.

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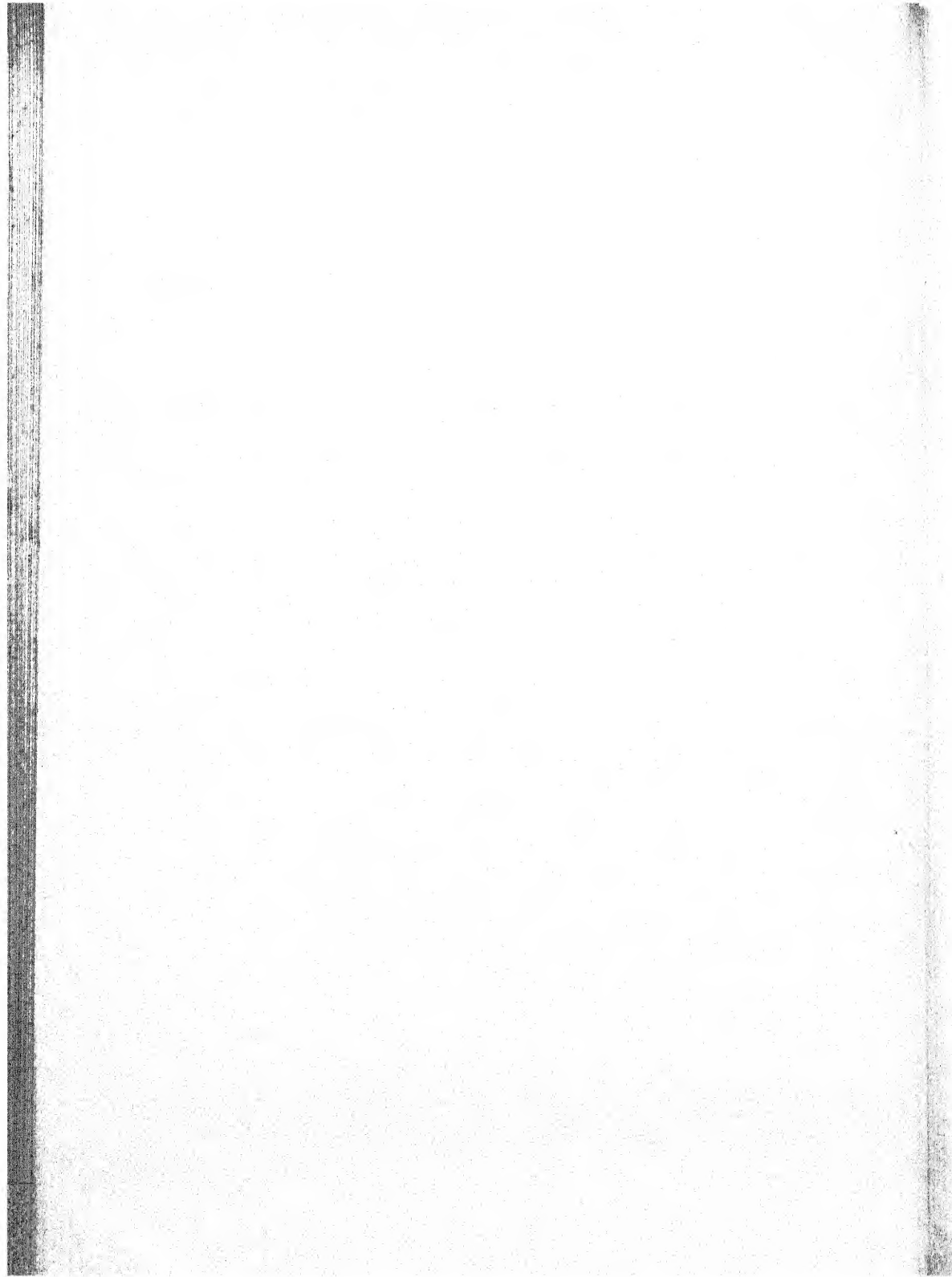
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X-RAY STUDIES OF THE STRUCTURE OF COMPOUNDS OF BIOCHEMICAL INTEREST

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INTRODUCTION

Since the publication of the last paper on this subject in the *Annual Review of Biochemistry* (1), x-ray diffraction methods have been applied, with varying degrees of success—as judged by the direct applicability of the conclusions to biochemical problems—to a large number of biochemically interesting substances. More important than the directly applicable results, however, is the accumulation of a background of knowledge concerning the details and principles of molecular and crystal architecture and concerning experimental methods. This is sure to prove of great value in extending detailed structure studies to the more complex substances in which biologists and biochemists are primarily interested. The technique of structure analysis has now reached such a stage that, given good crystals (of, say, a few tenths of a millimeter in average “diameter”), the structural details of even very complicated molecules can be deduced.

Especially useful for such work are the reliable data on interatomic distances—both between atoms which are directly bonded together and between nonbonded pairs of atoms—and on interbond angles, which have come from painstaking investigations of the structures of many substances composed of small molecules.

Hand in hand with the accumulation of this type of information has come an increased understanding of the principles determining the spatial distribution of atoms and molecules in crystals and other condensed systems. Three of these principles, which are of considerable importance in biological systems, have been chosen for special consideration here.

The environment of ions.—In salts, each ion tends to be surrounded by ions of opposite sign and, if these surrounding ions are polyatomic, to have as immediate neighbors those atoms which have the greatest excess effective charge of the opposite sign (resulting either from permanent polarization of bonds or from a high polariza-

bility of the atom concerned). The usual structural formula for a salt, according to which the number of bonds joining each metal or halogen ion (for instance) to other atoms equals its valence, does not represent the structure accurately (2, 3).

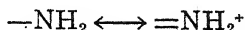
Many examples to illustrate this might be cited. One of those studied most recently is Rochelle salt, sodium potassium tartrate tetrahydrate, whose complicated structure has been worked out by Beevers & Hughes (4). In this compound each sodium ion, for instance, has as close neighbors two carboxyl oxygens (in different tartrate ions), one hydroxyl oxygen, and three oxygens of water molecules.

Another interesting example is afforded by glucosamine hydrobromide, the crystal structure of which has been reported by Cox & Jeffrey (5). Each bromide ion is surrounded, at distances suitable for $\text{N}-\text{H}\cdots\text{Br}$ and $\text{O}-\text{H}\cdots\text{Br}$ bridges, by one nitrogen (of an NH_3^+ group) and three oxygens (of hydroxyl groups). Incidentally, here (as in all other ammonium compounds of known structure) the ammonium nitrogen atom is not directly bonded to more than four other atoms. Its fifth valence represents a charge transfer (the formation of a pair of ions), rather than an electron-pair bond (6, 7).

Residual van der Waals forces.—Whether or not molecules and atoms attract each other as a result of ionic forces or hydrogen bridge forces (to be considered below), there are in all cases residual "van der Waals forces" between the atoms. These are usually attractions, except when the atoms are forced closer together than their characteristic equilibrium distances. They cause the molecules and their component atoms and groups to pack together as closely as possible, consistent with the satisfaction of the stronger forces mentioned, without bringing any atoms so close together as to produce strong repulsion. As already noted, our knowledge of the usual distances for equilibrium between atoms not directly bonded together is continually increasing, making it possible to limit more and more the structures to be considered for complicated substances. Advances in this field have been numerous and substantial.

Hydrogen bridges.—If suitable proton-sharing groups and proton-attracting atoms are present in a molecule, "hydrogen bridges" (or "hydrogen bonds") (8, 9, 10, 11) will be formed, either between different parts of the same molecule or between different molecules. The most important proton-sharing groups in the systems under discussion here are hydroxyl groups, carboxyl groups, substituted am-

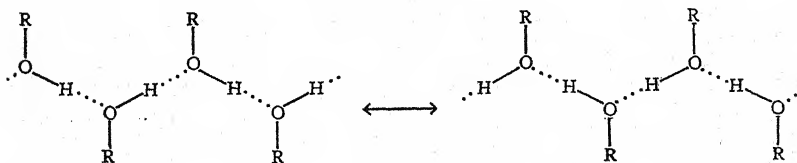
monium groups ($-\text{NH}_3^+$, etc.), and amino groups which are so situated (as in polypeptides) as to permit resonance (11) of the sort¹



The proton-attracting atoms which are important for our purpose are oxygen (in ketones, alcohols, acids, etc.) and nitrogen (in amines, not in ammonium compounds), the essential requirement being the presence of an available "lone pair" of electrons (12, 13).

The formation of hydrogen bridges is proving to be of great importance in determining the distribution of atoms in organic molecules—insofar as it is not determined by the distribution of ordinary covalent bonds—and between organic molecules, in the solid and liquid states (10, 11, 14, 15).

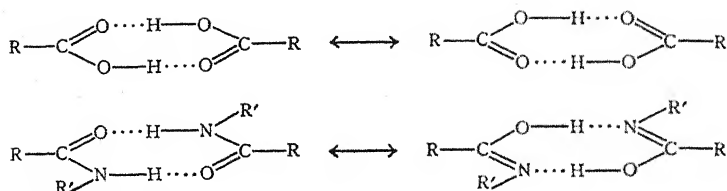
Rings and long chains of hydrogen bridges are especially stable, both because of resonance and because of the possibility (16) of synchronized oscillation of the bridging hydrogens and the electron systems between them. This is illustrated by the chains or rings of bridges of the sort



found to exist in all alcohols and phenols whose crystal structures have so far been determined—e.g., in pentaerythritol (17, 18), resorcinol (19, 20), and polyvinyl alcohol (21).

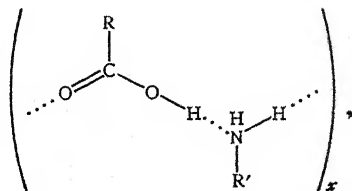
This increased stability also applies to rings and long chains in which the bridges are separated by other atoms and bonds, provided the resonance and synchronized oscillations are not thereby prevented. Typical resonating ring systems are the following:

¹ The double-headed arrow distinguishes (10) a state of electronic resonance from a state of chemical equilibrium, represented by \rightleftharpoons .



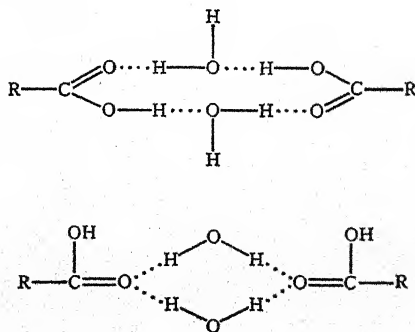
The first of these occurs in formic (22), oxalic (23), succinic (24), and other acids. The other is found, for example, in isatin (25) and in diketopiperazine (26).

Glycine (10, 27) and alanine (28) contain chains of bridges having the structure

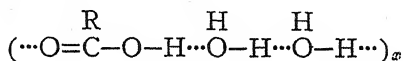


in resonance with several others, obtained by suitable shifts of the =, —, and ... bonds.

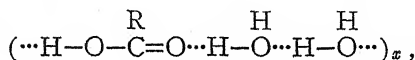
Crystals of hydrates of carboxylic acids and their salts contain rings or long chains of hydrogen bridges involving both the carboxyl oxygens and the water oxygens. Oxalic acid dihydrate (16, 29, 30, 31, 32), for instance, contains rings of the following two kinds:



The Rochelle salt structure (4) contains rings and chains of bridges of several sorts. The abnormally large value of the dielectric constant in one direction in these crystals may be associated, as Beevers & Hughes have pointed out, with the possibility of synchronous shifts of the hydrogens in these bridge systems, the impressed electric field causing the structure



to shift to



for example.

The reviewer is tempted to speculate here about the possibility that nerve conduction involves the shifting of hydrogens along similarly balanced chain systems. With this mechanism a slight increase in hydrogen ion concentration at one end of a bundle of chains could cause the liberation of hydrogen ions at the other end without any actual transfer of ions through the chains.

FIBROUS PROTEINS

To assist in solving the problems of protein structure, we now have available the results of careful, apparently accurate structure analyses of such small molecule crystals as those of glycine (27), alanine (28), diketopiperazine (26), acetamide (33), dicyandiamide (34), melamine (35, 36), urea (37), and the addition compound of urea and hydrogen peroxide (38). From these it seems safe to draw the following conclusions:

(a) In polypeptide chains or rings, $\left(\begin{array}{ccc} \text{H} & \text{R} & \text{O} \\ | & | & | \\ -\text{N}- & \text{C}_1- & \text{C}_2- \\ | & & \\ \text{H} & & \end{array} \right)_x$, the dis-

tances between adjacent atomic centers will be (within about 0.02 Å) as follows:

N to C₁, 1.41 Å; C₁ to C₂, 1.52 Å; C₂ to O, 1.25 Å; C₂ to N, 1.33 Å; C₁ to H, 1.09 Å.

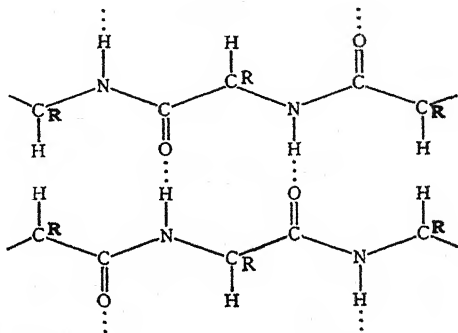
(b) The angles between adjacent bonds in the chain will be (within about 3°) as follows:

$$\angle \text{NC}_1\text{C}_2, 112^\circ; \angle \text{C}_1\text{C}_2\text{N}, 118^\circ; \angle \text{C}_2\text{NC}_1, 118^\circ.$$

(c) The chain nitrogen atoms and the oxygens attached to C_2 will be connected together by hydrogen bridges, of length (N to O) averaging $2.85 \pm 0.05 \text{ \AA}$. [The x-ray evidence for NHO bridges in proteins has been confirmed by infrared studies (39, 40).]

From the foregoing, structures can be deduced for the fibrous proteins which are in good agreement with the x-ray data.

Silk and β (stretched)-keratin seem to contain polypeptide chains stretched out nearly as much as possible (41, 42, 43), consistent with the bond distance and angle requirements; adjacent chains appear to be held together into two-dimensional nets by means of NHO bridges (10):



Here CR denotes a bond (to the first carbon atom of the R group) pointing up, while CR represents a C to R bond pointing down. In keratin, cystine radicals ($-\text{CH}_2-\text{S}-\text{S}-\text{CH}_2-$ replacing two R) may bridge between adjacent chains in two different nets, as assumed by Astbury and co-workers, or they may form bridges between adjacent chains in the same net. The interchain distances and C—R bond directions are satisfactory in either case. It is reasonable to suppose, too, that neighboring chains are also joined together through oxygen-containing bridges (e.g., $-\text{CH}_2-\text{O}-\text{CO}-\text{CH}_2-\text{CH}_2-$), such as would be produced by condensation together of the R groups of serine and glutamic acid.

In α (unstretched)-keratin, the polypeptide chains seem to be folded or coiled in such a way that the average extension per amino acid residue in the direction of the chain axis is about half as large as in β -keratin (42, 43). For reasons which Neurath & Bull (43a, 53) and the writer (44, 45) have presented in detail elsewhere, none of the

four structure patterns which Astbury and others have proposed (42, 43, 46, 47, 48, 48a) for α -keratin is entirely satisfactory. Instead, the writer proposes (44, 45) the distribution of bonds within each chain indicated in Figure 1a.

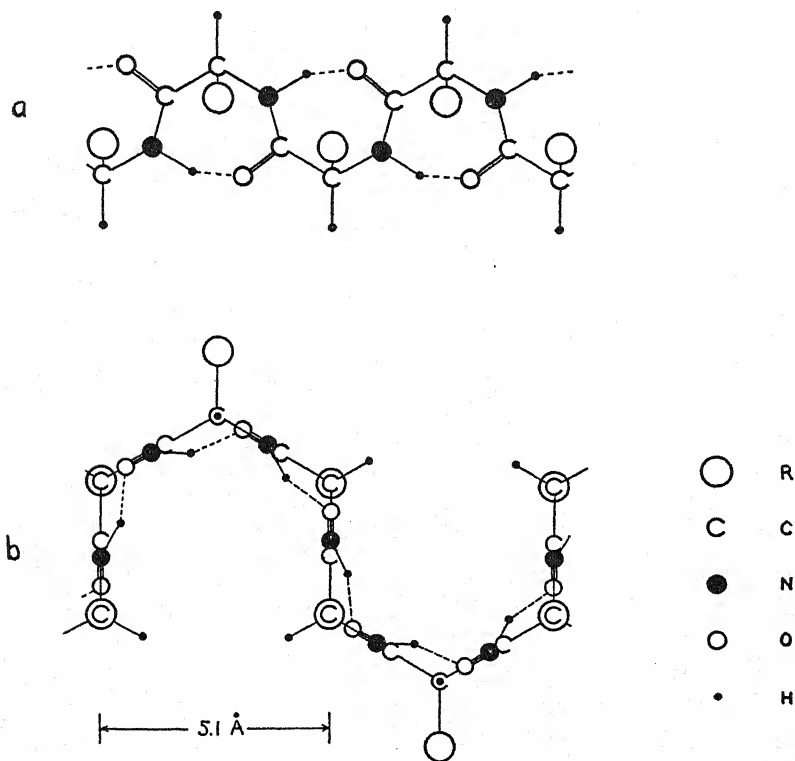
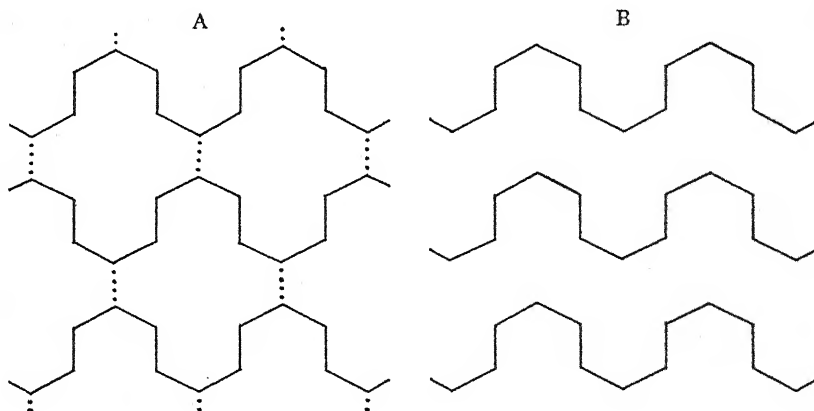


FIG. 1.—Representing a possible structure for a polypeptide chain in α -keratin.

If suitable bond angles are to be maintained, this ribbonlike structure cannot be plane but must be folded. One plausible type of folding, agreeing with the x-ray data, makes the chain a spiral having a projection (on a plane normal to the ribbon plane) as represented in Figure 1b.

These spiral chains would be expected to align themselves in layers in one of the following ways:



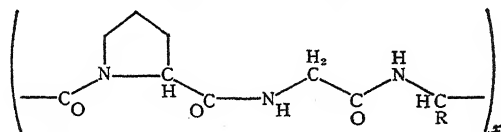
If arrangement A is the correct one, adjacent chains in each layer are probably connected by disulfide and other bridges as indicated by the dotted lines. If B is correct, such bridges probably connect adjacent chains in different layers. In either case, the transformation from α - to β -keratin requires no breaking of these bridges.

On the other hand, some bridges connecting R groups—perhaps in the same chain—must be broken in the transformation from α - to β -myosin, if we follow Astbury (49) in assuming a close similarity between the myosin and keratin structures.

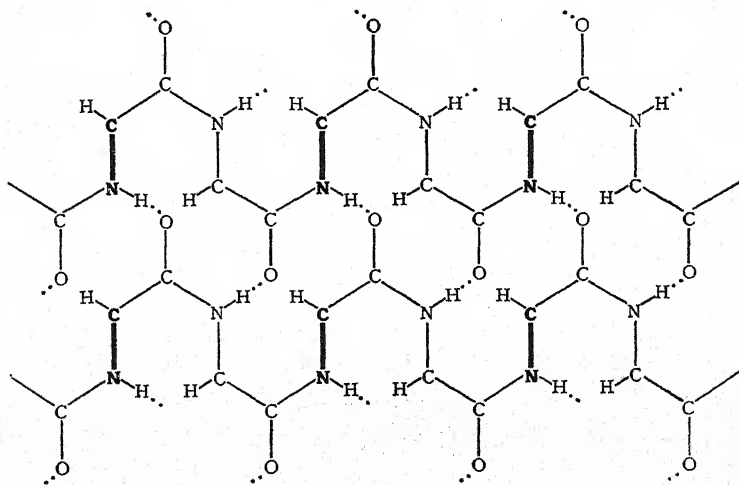
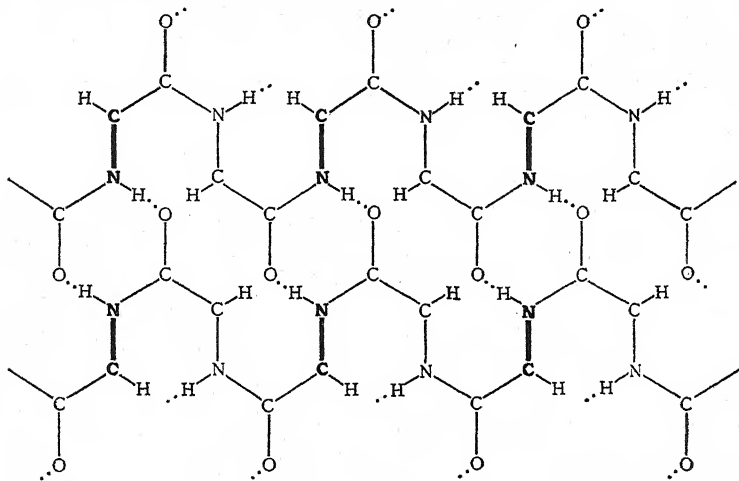
Supercontracted keratin may, as Astbury supposes, have a regular structure in which the individual chains are folded in larger folds than in the α -form. Other alternatives to be considered, however, are that the β -keratin net described above be folded without rupture, in either a regular or irregular way, and that the chains be much kinked and disoriented, forming hydrogen bridges where possible but in quite a random manner. According to this last picture, the supercontracted keratin structure would resemble that of unstretched rubber, except that the forces holding different chains (and neighboring bends in the same chain) together would be much stronger. Careful studies of the relationships between tension and extension at different temperatures would help to decide how much of the elasticity in this and related cases is truly rubberlike—a result of differences in randomness between the unstretched and stretched states.

Similar remarks apply to supercontracted myosin, with the exception that folding of α -myosin layers would seem to be more probable than folding of β -myosin nets.

For collagen chains, Astbury (50, 51) has proposed two different types of structure. The more recent can be represented by the formula



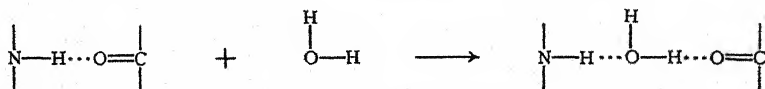
Every third residue is assumed to be either proline or hydroxyproline. For reasons which will be discussed elsewhere (45), the reviewer prefers either of the two structures represented below:



The R groups are not shown in these formulas. They are directly under the carbon atom (of the CH groups) represented by C and over those represented by **C**. Each chain is a spiral, the uppermost atoms being represented in heavy type. The proline and hydroxyproline residues, which together constitute nearly one third of the total (48), can be accommodated much more readily than in the structure suggested above for α -keratin. The proline rings extend either above or below the net shown. For each such ring, there must obviously be one less interchain hydrogen bridge, unless a hydrogen ion is supplied from a hydroxyl group (e.g., in the hydroxyproline residue). Such a zwitterion structure, in which the proline nitrogen atom has a formal charge (3) of $+1$ and a hydroxyl or carboxyl oxygen has a formal charge of -1 , is by no means unreasonable.

If either of these two models correctly represents the collagen structure, gelatin would be expected to be similar, but less regular, as a result of the fission of many of the NHO bridges between the chains, followed or accompanied by the formation of new bridges in other directions. Considering both NHO bridges and ordinary covalent bonds as determining the relative positions of the atoms, gelatin (in the absence of much water) can be described as having an irregular, three-dimensional network structure, whereas collagen is—except for junctions through the R groups—an assembly of parallel two-dimensional nets.

When collagen or gelatin absorbs a small amount of water, the water molecules presumably insert themselves between the ends of the most accessible hydrogen bridges, in this way, for example:



"Bound water" in isoelectric gelatin is presumably held largely in this manner. As more water is absorbed, water-to-water bridges must be formed. A water molecule held only by such bridges is in nearly the same environment as in pure water.

Elastin has a large proline content and but little hydroxyproline or other component capable of furnishing the hydrogen ions needed for NHO bridges from the proline nitrogens (52). The chains should therefore have a greater freedom of orientation than in collagen, with greater flexibility (due to fewer NHO bridges) than gelatin. Although elastin may contain regions in which a collagen type of struc-

ture exists, it seems probable that the chain distribution is, on the whole, quite random, consistent with the existence of NHO bridges wherever possible. To this randomness and the possibility of considerable freedom of rotation about many of the chain bonds may be attributed its long-range, rubberlike elasticity.

It is worth noting that in the structures suggested above for α -keratin and for collagen there is no crowding of the R groups. They lie alternately above and below the plane of the net, in each case, at quite reasonable distances apart. Neurath's (53) objections to certain other proposed structures therefore do not apply to these.

CORPUSCULAR PROTEINS

Although the structural details of no "globular" or "corpuscular" protein are yet known, a considerable amount of potentially useful x-ray data has been accumulated (54, 55). Not counting the work on virus proteins, the size, shape, symmetry, and mass in molecular weight units (together with the probable number of molecules) of the unit cell have been determined for dry (56) and wet (55, 57) insulin, lactoglobulin (55, 58), chymotrypsin (54, 59, 60), pepsin (61), horse methemoglobin (55, 59, 60), tobacco seed globulin (62), and excelsin (63, 64). Patterson interatomic vector projections and sections have been prepared from the x-ray data on insulin (both dry and wet), lactoglobulin, and horse methemoglobin. Attempts have been made by Wrinch (65 to 73) to show that the main features of some of these projections and sections can be deduced from certain hypothetical types of structure, but all x-ray workers in this field (at least all who have expressed themselves in print) seem to be agreed that little can be concluded from the present x-ray data regarding the correctness or incorrectness of the assumed structures, and that such evidence as these data do furnish (as well as evidence from other sources) is definitely against Wrinch's hypotheses (55, 74 to 86). More x-ray data are obviously needed—especially data (e.g., from two different metal salts of a protein having suitable crystal symmetry) which would enable the determination of the phases of the x-ray reflections from planes in one or more of the principal zones (76). From the phases and intensities, electron density projections could then be prepared, and from these one could hope to deduce the details of the atomic arrangement in the structure.

The unit cell sizes, shapes, and symmetries of course limit the molecular configurations considerably. The x-ray results furnish a

check on deductions regarding molecular weights and shapes from ultracentrifuge, viscosity, and diffusion data—deductions based on a less sound theoretical foundation than the x-ray conclusions. This phase of the subject, however, need not be further discussed here since it is adequately dealt with elsewhere (55, 87, 88, 89).

Wrinch (65, 66, 72, 73) has shown that certain two-dimensional fabrics which she has considered as possible models for protein structures can be folded in such a way as to give closed polyhedra of various shapes (e.g., octahedra) but only certain ("quantized") sizes, as measured by the number of amino acid residues. On this basis, she explains the supposed approximate quantization of molecular weights of globular proteins. [The evidence for and against such quantization is reviewed by Edsall (89) in this volume.]

This ability of a regular pattern to fold to give closed structures of quantized sizes is, of course, not peculiar to Wrinch's "cyclol" and related fabrics (86, 90). For example, the two-dimensional nets suggested above for β - and α -keratin and for collagen (as well as other nets which the reviewer has considered) can be folded to give structures, such as the open-ended prisms and octahedra depicted in Figure 2, in which the polypeptide chains are closed to form rings (45, 86).

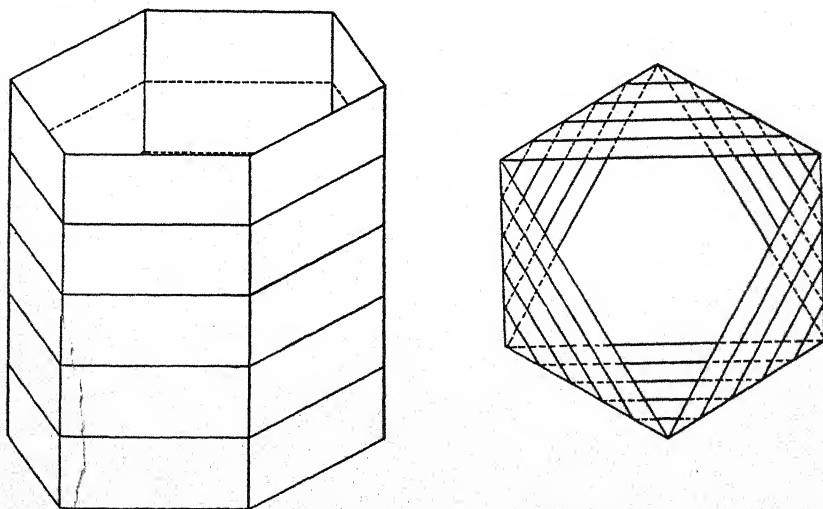


FIG. 2.—Illustrating the distribution of polypeptide chain axes in hypothetical molecules derived by folding from structures such as those described for keratin and collagen.

Folding of the second pattern suggested for collagen, for example, can produce an open-ended octahedron consisting of eight rings of thirty-six residues each, which is at least approximately of the right size, shape, and symmetry to fit into the insulin unit cell.

By accompanying the folding with a slight shift of the pattern, normal to the chain axes, one can produce, instead of a structure composed of polypeptide rings, one in which the whole "globule" consists of a single polypeptide chain. This picture has much to recommend it, though it supplies no obvious reason for the quantization of residue numbers.

Protein denaturation certainly involves the breaking of hydrogen bridges or other types of bonds between the chains, presumably with an increase in randomness (91) of the spatial distribution of the R groups and probably of the chains themselves. X-ray studies by Astbury and co-workers (63, 92, 93) and by Spiegel-Adolf & Henny (94, 95), however, show that in certain instances some of the powder diffraction lines are actually sharpened and new lines appear. This seems to indicate an increase in regularity, at least in portions of the structure or as regards certain features of the structural pattern. Perhaps, as Astbury suggests (63), denaturation always involves an approach to a β -keratin type of structure. Further work is obviously needed before such a hypothesis can be considered established and before the real significance of the experimental results can be understood.

VIRUS PROTEINS

The brilliant work of Bernal & Fankuchen on tobacco mosaic and other viruses, only the outlines of which had been published at the time Astbury wrote his review (1) for Volume VIII of this series, has since been published in detail (96). The x-ray data on tobacco mosaic virus show it to be composed of rodlike particles having a diameter of about 150 Å. By other methods, the length has been shown to be about 1500 Å or a multiple thereof. The virus forms anisotropic gels and solutions in which the rods are placed end-to-end in rows, with the rows parallel to each other in the hexagonal pattern which would be expected if they all possessed large electrical charges of the same sign and so mutually repelled each other. An equivalent amount of charges of the opposite sign must, of course, be carried by small ions in the solution or gel between the rods. Further studies of systems like this

by x-rays and other means should be useful in the development of the theory of the nature and properties of gels.

As for the internal structure of the rodlike particles, a unit cell has been deduced, and there has been some speculation regarding the possible existence and distribution of subunits of dimensions $44 \times 44 \times 22 \text{ \AA}$, with "sub-subunits" of dimensions $11 \times 11 \times 11 \text{ \AA}$.

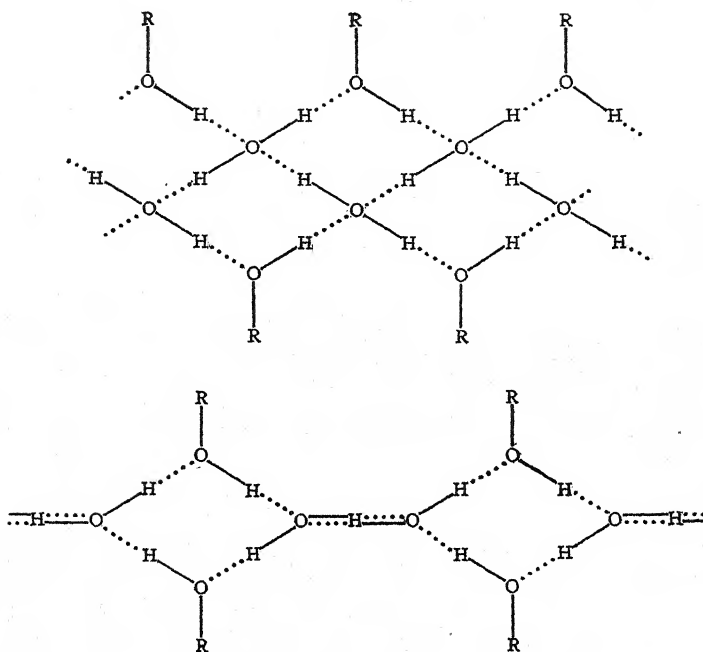
Some x-ray data have also been obtained from other viruses, but the interpretation has not been carried so far as in the case of tobacco mosaic virus.

STEROLS AND RELATED COMPOUNDS

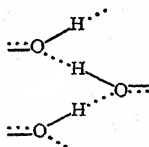
Bernal, Crowfoot & Fankuchen (97) have published an extensive preliminary x-ray study of a large number of sterol derivatives. They record the unit cell dimensions and space groups, as well as data on optical properties and crystal habits, for some eighty compounds. In three instances (cholesteryl chloride, cholesteryl bromide, and cholesteryl chloride hydrochloride) they have proceeded further, making Patterson interatomic vector projections. These projections show the molecules to be roughly lath-shaped ($20 \times 7 \times 4 \text{ \AA}$) and define their approximate relative positions and orientations in the crystals. Moreover, certain details of the patterns can be correlated with certain features of the molecular structures, as assumed on the basis of purely chemical evidence.

With the information outlined above, these compounds, together with some twenty-five others previously studied by the same or other authors, have been classified on the basis of similarity of arrangement of the molecules in the crystals. The attempted correlation of this classification with the molecular formulas has not been very successful. It has been shown, though, that the presence of a hydroxyl group attached to carbon atom number 3, at one end of the sterol skeleton, is generally associated with a "double-layer" structure in which the hydroxyl ends of the molecules in one layer are adjacent to the hydroxyl ends of the molecules in one of the two adjacent layers; i.e., the two layers of each pair are face-to-face.

As the authors suggest, adjacent terminal hydroxyl groups of each pair of layers are probably connected—perhaps through water molecules—by hydrogen bridges. To make the picture more definite, the reviewer suggests, as possible alternatives for the cases in which there is one water molecule per sterol molecule, the two arrangements shown below (somewhat idealized, for representation in two dimensions).



In the latter formulation, $\text{O} \cdots \text{H} \cdots \text{O}$ represents the projection of a zigzag chain of bridges connecting oxygen atoms of water molecules:



Bernal, Crowfoot & Fankuchen show that data such as they have collected are useful for identification and classification of sterol derivatives and for the determination of molecular weights and numbers of molecules of water of crystallization. As they point out, however, most of the outstanding problems concerning the molecular structures can be settled only by more detailed analysis. This survey promises to be of great help as a guide for future investigations, supplying the necessary information for deciding which compounds can most profitably be studied more intensively.

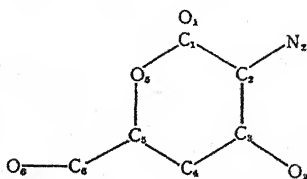
X-ray crystallographic measurements by Carlisle & Crowfoot (98) have been used to establish the molecular symmetry of a number of

compounds of the diethylstilbene and diethylbenzyl series. The arrangement of atoms in the *meso* series, which is biologically the most active, is very closely related to the stereochemical form which has been deduced for the natural sex hormones. The presence of a molecular center of symmetry proves that the disposition of the atoms about the central C—C bond is of the *trans* type considered characteristic of the junction between rings B and C of the sterol sex hormone series.

CARBOHYDRATES

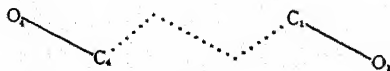
Probably the most important recent work in the carbohydrate field is that of Cox & Jeffrey (99) on the structures of α -chitosamine hydrochloride and hydrobromide. This is the first instance in which the configuration of a carbon compound containing several asymmetric centers has been confirmed by direct determination of the atomic positions.

Omitting the hydrogens, the chitosamine ion can be represented by the following formula:

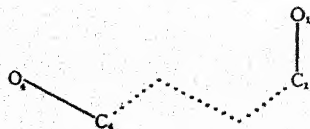


The pyranose ring is slightly puckered. Atoms C₂, C₄, O₅, O₆, and C₆ are approximately in the same plane—say, the plane of the paper. Then C₁, C₃, C₅, N₂, and O₄ are approximately in another plane, slightly above the plane of the paper, and atom O₁ is still higher, practically directly over C₁.

This work verifies the relative orientation of the groups in α - and β -glucose derivatives. In cellobiose and cellulose, which are β -derivatives, the relative orientation of the C₁—O and C₄—O bonds is



whereas, in the α -chitosamine ion, in α -maltose, and in starch, it is



No other thorough structure investigations, yielding atomic positions, of carbohydrates or their derivatives have yet been reported, though the unit cell dimensions and symmetries have been derived in quite a few cases. For the older work a review by Mark (100) may be consulted. Recent work includes studies of some maltose derivatives by French & Bear (101) and of cellobiose and cellotriose esters by Ohasi (102).

Bear & French (103) have recently reported the results of a new x-ray study of starch. In spite of the apparent impossibility of obtaining either single crystals or oriented fibers of this substance, they have been able to deduce the dimensions of the unit cells—one for corn starch, the other for potato starch. These cells differ from each other only slightly in size and shape, although the x-ray patterns exhibit very marked differences. Each unit cell probably contains four glucose (two maltose) residues.

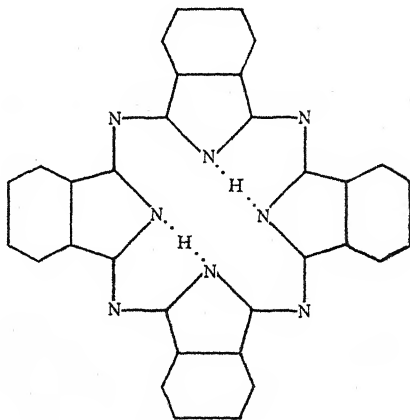
Astbury, Bell & Hanes (104) and Bear & Cori (104a) have reported that synthetic starches prepared by the action of phosphorylase on glucose-1-phosphate give x-ray patterns essentially the same as those from natural starches.

The dispute (105, 106) concerning the x-ray data from cellulose now seems to be settled (107, 108). Meyer & Mark's modification of the Sponser & Dore structure (110) must be a close approximation to the truth. Certain weak reflections remain unaccounted for by this model, however, unless one assumes some deviation from perfect regularity of structure, such as a lack of complete regularity in the directions of the chain axes or in the orientation of certain groups. The atomic positions within the unit cell remain unknown. A new attack on the problem, using recently developed methods, together with the information furnished by the chitosamine study and our present knowledge of hydrogen bridges in other hydroxy compounds, would doubtless yield the complete picture.

The microscopic and x-ray studies of Preston & Astbury (1, 111) on the cell wall of *Valonia* have been extended to several species of the filamentous green alga *Cladophora* (112). The cellulose chains are again found to form spirals, those in adjacent layers alternating in direction and in pitch. This work constitutes an important step toward a more detailed knowledge of the mechanism of plant growth.

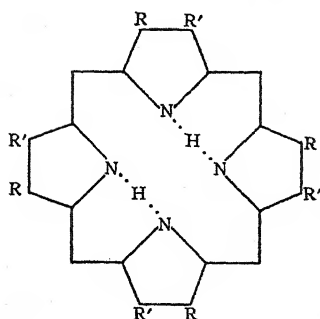
PHTHALOCYANINES AND PORPHINES

The applicability of modern x-ray methods to the determination of the structural details of complex molecules has nowhere been more beautifully exemplified than in the researches by Robertson and co-workers (113 to 117) on phthalocyanine and its salts. Most recently, they have reported on the platinum salt; they give the positions of all the atoms in the molecule (except hydrogen) within an estimated average accuracy of about 0.05 Å. These studies furnish most convincing evidence of the reality of the concept of resonance (11) between structures differing in the distribution of double and single bonds but not (appreciably) in the distribution of atomic centers. The structural formula for phthalocyanine can be represented as shown below.



Each full line here represents a bond which (because of resonance) is intermediate between a single bond and a double bond. Each half of a hydrogen bridge (of the unusual NHN variety) is represented as a dotted line.

A preliminary report on the closely related structures of tetrabenzoporphine and tetrabenzomonazaporphine, which have structural formulas like that of phthalocyanine except for the replacement of four and three, respectively, of the outer nitrogen atoms by CH groups, has been published by Woodward (118). Christ & Harker (119) have also presented a preliminary report on a structural study of etioporphyrin I, which can be represented by the following formula:



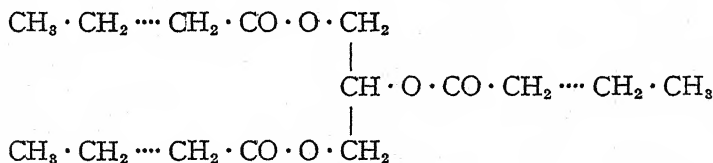
where $R = \text{CH}_3$ and $R' = \text{C}_2\text{H}_5$.

Continued work in this field is important because of its bearing on the chlorophyll and hemoglobin problems.

GLYCERIDES

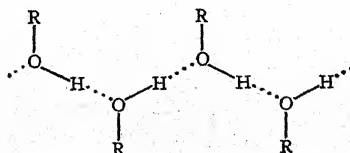
Comprehensive x-ray and thermal studies of mono-, di-, and triglycerides have been reported during the past few years by Malkin and co-workers.

In crystals of the triglycerides (120, 121, 122) the molecules apparently have a "tuning fork structure," as indicated by the formula

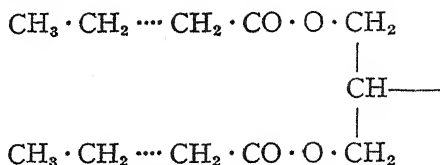


The chains in each branch are of course zigzag, not straight.

Crystals of the α, α' -diglycerides (123) have a "double-layer" structure like the normal alkyl alcohols and acids and the sterols having a terminal hydroxyl group. The two layers of each pair may be assumed to be held together in this fashion,



where R represents



The α -monoglycerides (124) also have a double-layer type of structure. We may reasonably assume that adjacent layers are here also held together by hydrogen bridges in a somewhat similar manner.

CONCLUSION

With a sound background of adequately tested structural principles, with the very powerful methods of structure analysis now available, and with the knowledge resulting from preliminary studies of many substances of biochemical interest and importance, we can look forward with confidence to great advances in this field in the future. How fast such advances will come will depend largely on the availability of money and of adequately trained personnel for this work.

In conclusion, the writer is glad to express his indebtedness to Drs. H. B. Bull, D. R. Goddard, H. Neurath, F. O. Schmitt, and S. E. Sheppard for helpful discussion of some of the topics dealt with in this review.

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HYDROLYTIC ENZYMES, NONPROTEOLYTIC

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CARBOHYDRASES

Amylases.—Clarification of an ambiguous situation with regard to the action of amylase on oxidized starch was undertaken by Örtenblad & Myrbäck (1). Myrbäck had shown originally that intensive oxidation of starch or dextrin by alkaline iodine did not affect hydrolysis by malt amylases, a finding compatible with the data indicating that saccharogenic amylase attacks the free nonreducing ends of starch chains. However, Mayer (2) claimed that, when starch is treated with iodine in quantities just insufficient to give a blue color, it is rendered resistant to saccharification although it can still be liquefied by enzyme action. Repetition of Mayer's experiments by Örtenblad & Myrbäck showed that Mayer was actually not dealing with oxidized products of starch since his solutions were neutral or even acid, and the resistance to saccharification which Örtenblad & Myrbäck observed in the presence of excess iodine could be attributed to a poisoning effect since sodium thiosulfate restored the enzyme activity. When the amount of iodine employed was insufficient to produce a blue color, the hydrolytic effect of the amylase could be demonstrated in the absence of thiosulfate. A study of oxidation and reduction effects on the actions of α - and β -amylase, amylosynthase, and sucrase was made by Itō (3) who found that hydrolytic activities are accelerated by reduction and retarded by oxidation, while synthetic activity is influenced in the reverse manner.

The interesting fact that thiourea tastes differently to different people led Drozdov & Sklyarov (4) to an investigation of the influence of arylthioureas on the activities of salivary and malt amylases, and takadiastase. They reported acceleration of enzyme action of approximately the same magnitude in the presence of equal concentrations of phenyl-, *o*-tolyl-, or *p*-tolylthiourea.

The effects of different buffers on the activity of β -amylase from wheat were studied by Ballou & Luck (5) as an extension of their previous work on takadiastase. Variation of the buffer anion had little effect on the pH optimum which ranged from 4.7 to 5.3 for different buffers, and the activity-pH curves coincided on the alkaline

side, but diverged on the acid side of the optimum. Relatively high concentrations of urea (2 to 4*M*) were found to inhibit the saccharogenic action of the enzyme on starch. In this connection it is interesting that Takano (6), in a study of the glucolytic action of lung tissue, reported that dilute urea solutions (one part per thousand) accelerated the enzyme effect. Without implying that the two enzyme systems are at all related, it should be borne in mind that it is not uncommon for a substance that activates a hydrolytic enzyme reaction in low concentrations to inhibit the same reaction in higher concentrations. Takano also observed a pH optimum of 4.5 to 5.2 for the glucolytic action of lung tissue. Yamagisi (7) found a pH optimum of 4.0 to 4.5 for the amylase of germinated rice purified by alumina adsorption.

A study of the kinetics of the breakdown of starch by β -amylase was made by Meyer & Press (8) who found evidence for the existence of an intermediary enzyme-substrate compound with a dissociation constant of 0.0008 when potato starch with 20 to 25 terminal glucose groups was employed as the substrate. The maltose formed by the hydrolysis had an inhibitory action on the enzyme. A study carried out on glycogen from beef liver and mussels indicated a similar hydrolytic mechanism, and the dissociation constants were of a greater magnitude (9). Lyoglycogen, prepared from fresh-water mussels, formed a protein-glycogen complex that was fully resistant to the action of β -amylase, but when the protein was precipitated with the Folin-Wu reagent, the carbohydrate was split more rapidly than the other glycogens of liver or mussels. The role of amylase in glycogen breakdown by pancreas (10), liver (11), and mussel hash (12) has been the subject of other investigations.

A number of papers have appeared dealing with amylase in grain as a function of variety, climatic conditions, germination, etc. From their study, Tyurina & Vukolikova (13) concluded that the preference of winter wheat and rye for bread baking over the spring varieties may be accounted for by the higher diastatic activities of the former. An interesting attempt at characterizing various wheat species of known chromosome constitution by the activity and nature of their amylase has been made by Knyaginichev *et al.* (14). Mansyreva (15) investigated the amylase and catalase activities in the leaves of spring wheat in relation to drought resistance. Drought conditions effected a decrease in amylase activity but did not influence the catalase, a finding in accord with that of Popova (16). The former author

has made the claim that not only are the amylase activities subject to variations with regard to the variety of the wheat and the season of the year, but also with the time of day, a maximum being reached at 10:00 A.M. and a minimum at 4:00 P.M. Burkert & Dickson (17) studied the action of the amylase of two barley varieties on the barley starches.

Another paper appeared by Myrbäck & Örténblad (18) concerning their controversy of several years standing with Chrzaszcz & Janicki on the mechanism of the conversion of inactive amylase in barley to the active form. Myrbäck & Örténblad have championed the view that in the inactive state the enzyme is bound to protein from which the active form may be liberated by proteolytic enzyme action; they have sharply attacked the theory of the Polish workers who held that a natural inhibitor of amylase, "sisto-amylase," exists in the grain which is counteracted by peptone set free through protease action. The weight of evidence would appear to favor the stand maintained by the Swedish investigators. Dull & Swanson (19) have presented evidence that the release of bound amylase in barley by salt solutions is the result of peptization of the protein to which the enzyme is attached.

The increases in amylase of wheat and barley during germination were followed by Popov (20) and Mayer & Klinga-Mayer (21) respectively. Cytological studies by Nezgovorov on the amylase in plastids separated from the cytoplasm of leaves indicated that only insignificant amounts of free (22) or bound (23) amylase resided in these elements. No increase in the enzyme activity was observed when the plastids were disintegrated by freezing.

The histological distribution of amylase in the rabbit kidney was determined by Weil & Jennings (24) using the apparatus of Linderstrøm-Lang & Holter. They observed that activity was localized in the cells of the proximal and distal convoluted and collecting tubules, while the cells of the loops of Henle were without activity.

Studies have been carried out to determine the possible diagnostic value of the measurement of amylase in blood serum. Popper *et al.* (25) observed elevated blood amylase levels in dogs with active pathological processes of the pancreas and concluded that the longer the enzyme level remains abnormally high the greater the severity of the disease. Ligation of all pancreatic ducts resulted in a transitory rise of blood amylase in contrast to the permanent increase reported by others (26). The results of the dog experiments of Dozzi (27) may

indicate that the liver too is related to the amylase level since the hepatic damage caused by chloroform poisoning is accompanied by a decrease in the enzyme.

Recent investigations of Lewison (28) and Gray *et al.* (29) more firmly establish that subnormal serum amylase levels indicate liver disease while elevated values are suggestive of pancreatitis. However, it must be borne in mind that high serum amylase values are also obtained in diseases of the salivary glands, kidneys, and in perforating peptic ulcers. Furthermore the rise in serum amylase in pancreatitis reaches a maximum in about the first forty-eight hours and thereafter recedes to normal within a few days. Hence the enzyme test can be used to advantage as an aid in establishing a diagnosis of only the acute stage of pancreatitis, and then only after interfering conditions are ruled out. The content of salivary and pancreatic amylase in humans as a function of age was studied by Meyer & Necheles (30).

Starch and glycogen synthesis.—The classical work on enzymatic synthesis of starch and glycogen that has been a focus of interest during the past few years has been extended by a number of recent studies. Astbury, Bell & Hanes (31) were able to show that the same x-ray diffraction pattern is given both by native potato starch and the synthetic polysaccharide prepared from glucose-1-phosphate by potato phosphorylase. Bear & Cori (32) demonstrated that the synthetic polysaccharide resembling plant starches, that can be prepared *in vitro* with muscle phosphorylase, gives a different type of x-ray diffraction pattern than the glycogen-like product obtained with the heart and liver enzymes. No explanation has been forthcoming for the fact that muscle yields starch *in vitro* and glycogen *in vivo*, while liver, heart, and brain produce glycogen in both cases.

In spite of similarities between natural and synthetic starches, there exist certain differences such as the lower solubility of the latter in water, its more intense iodine coloration, and its quantitative hydrolysis by β -amylase to maltose. With natural starch, β -amylase action stops when about 60 per cent is converted to maltose. In order to determine whether these differences in behavior are related to molecular constitution, Hassid & McCready (33) made a study of the chemical structure of the synthetic product. By hydrolysis of the methylated starch and subsequent separation of cleavage products, they obtained 2,3,6-trimethylglucose as the sole hydrolytic product in contrast to the tetramethylglucose obtained from natural starches. This finding suggested that the synthetic molecule is made up either

of long chains in which anhydroglucose units exist in numbers too great to allow isolation of tetramethylglucose, or the chains are in the form of continuous loops.

The action of both plant and animal phosphorylases in bringing about the reversible formation of polysaccharide from the Cori ester (glucose-1-phosphate) has been demonstrated by various investigators (34 to 37). Sutherland, Colowick & Cori (38) have more recently established the reversible enzymatic formation of glucose-6-phosphate from the Cori ester by phosphoglucomutase, thus removing the chief obstacle in the clarification of the mechanism of glycogen synthesis from glucose and fructose. They have also succeeded in separating phosphoglucomutase from both phosphorylase and isomerase. These investigators report that the first three enzymatic reactions for glycogen breakdown in tissues at pH 7 and 25° are as follows:

- I. Glycogen + inorganic phosphate (77%) \rightleftharpoons glucose-1-phosphate (23%)
- II. Glucose-1-phosphate (6%) \rightleftharpoons glucose-6-phosphate (94%)
- III. Glucose-6-phosphate (70%) \rightleftharpoons fructose-6-phosphate (30%)

Colowick & Kalckar (39) found a heat-stable protein in muscle tissue that activates the hexokinase system capable of converting glucose and fructose to the respective 6-phosphates by transferring the labile phosphate groups of adenosine triphosphate to the hexoses.

Other related studies include an investigation by Colowick, Kalckar & Cori (40) of the mechanism of glucose phosphorylation and oxidation by cell-free tissue extracts; a confirmation by Wolfson & Pletcher (41) of the original suggestion by Cori *et al.* that the Cori ester possesses the constitution of a *d*-glucopyranose-1-phosphate; work by Macfarlane & Weil-Malherbe on phosphorylating glycolysis in brain slices (42); similar work by Ochoa on brain dispersions (43) and extracts (44); a study by Sakov (45) on the formation and hydrolysis of esters of adenosine phosphoric acid by muscle extract in the presence of Neuberger ester; and a report on starch phosphorylase by Green & Stumpf (46).

Hyaluronidase.—The great interest in this enzyme that has flared up within the last two or three years is attributable to its relation to the "spreading factor," an agent that increases tissue permeability and hence is involved in the invasive ability of infective materials. Since the original observation of Chain & Duthie (47) that testicular prep-

arations of the spreading factor exhibit a mucolytic effect by simultaneously lowering the viscosity of, and liberating reducing substances from, synovial fluid or vitreous humor, investigators have been concerned with the question of the identity of hyaluronidase and the spreading factor. Chain & Duthie themselves have been inclined to regard the enzyme and the factor as the same substance, basing their conclusions on quantitative comparisons of the two, using preparations from testes, leeches, and bee sting extracts (48).

Continuing their earlier work on this subject, Hobby *et al.* (49) have shown that all preparations containing hyaluronidase also contain the spreading factor; both are weakened by heating at 65° for thirty minutes, and almost completely inactivated at 100° in this interval; both are partially inactivated by iodine, and neither is reactivated by sodium sulfite under the conditions employed. It is significant that hyaluronic acid has been isolated from skin (50). However, certain data speak against the identity of the enzyme and the spreading factor: these authors (49) point out that many preparations possessing spreading properties have none of the enzyme activity, and antisera against pneumococcal hyaluronidase specifically and completely inhibit the homologous enzyme but not the spreading factor. On the other hand McClean & Hale (51) claimed that both viscosity reduction and liberation of reducing substances by bacterial enzymes can be completely neutralized by appropriate antisera which also inhibit diffusion in the skin. The latter investigators indicate that the failure of Meyer *et al.* to demonstrate inhibition of diffusion with pneumococcal antisera can probably be explained by the fact that antisera completely inhibit only a limited number of minimal diffusing doses of the factor in the skin. Whoever may eventually prove to be correct, the following possibilities presented in an admirable treatment by Hobby *et al.* (49) must be considered: (a) The spreading phenomenon may be produced by several agents of which the enzyme is but one; (b) differences observed between spreading and enzyme activity may be due to the fact that the former is measured *in vivo* and the latter *in vitro*; (c) differences between the two may also result from the greater sensitivity of the spreading factor test, although this is not likely from the lack of parallelism in the activity of many preparations; (d) skin itself contains a spreading factor thus raising the possibility that the effect is due to release of the factor by the injected material which would account for the spreading properties of simple substances like arsenious oxide.

Several publications dealing with the properties of hyaluronidase have appeared. Meyer *et al.* (52) investigated both viscosity-lowering and hydrolytic effects of the enzyme, and observed hydrolytic maxima at pH 4.4 and 5.8 for testicular preparations while those from bacterial sources exhibited only a single optimum (pH 5.8). The depolymerizing action of testicular preparations is more pronounced than that of pneumococcal sources, and the authors have interpreted these results as the effect of two enzymes, one that attacks the long chain molecule, and the other that hydrolyzes the aldobionic acid units which are probably formed at about 50 per cent of the total hydrolysis. They also found that hyaluronidase preparations hydrolyze the sulfuric acid containing polysaccharide of the cornea, and the testis enzyme attacks chondroitinsulfuric acid.

McClean & Hale (51) observed a pH optimum at about 4.6 for the viscosity-lowering activity of the enzyme from testis, bacterial filtrates, and the venom of Russell's viper in agreement with earlier work of Madinaveitia & Quibell (53); this value corresponds closely with the hydrolytic optimum reported by Meyer *et al.* (52). McClean & Hale also found that when sufficient enzyme was present to lower the viscosity in a few minutes to one half the starting value, no *N*-acetylhexosamine could be detected for some hours. They suggested that this finding, together with others showing a lack of correlation between viscosity reduction and liberation of reducing substances by different enzyme samples from the same source, points to the view that two separate mechanisms are involved in the polysaccharide breakdown. This is in accord with the suggestion made independently by Meyer *et al.* (52).

The effect of salt concentration on both viscosity-lowering and acetylhexosamine-liberating properties of the enzyme was investigated by several workers (51, 54, 55). With low salt concentrations the enzyme was activated by anions and inhibited by cations, while both inhibited at higher concentrations.

The extensive study by McClean & Hale (51) included the demonstration that the enzyme in Russell's viper venom is independent of the action the venom exerts on heparin, even though the latter is similar to hyaluronic acid. Addition of potassium hyaluronate to the culture medium of *Cl. welchii* increased the enzyme yield, thus demonstrating the adaptive nature of this strain. [Adaptive properties of the group B strain of *Streptococcus pyogenes* have also been shown in a later study by McClean (56)]. Agents such as azoproteins, as-

corbic acid, etc. that reduce the viscosity of mucoprotein solutions do not cause liberation of reducing substances, nor do they activate hyaluronidase; hence their effect must be of another nature. An investigation of these nonenzyme agents by Madinaveitia & Quibell (57) should be mentioned.

An interesting application of hyaluronidase that may prove to be highly important was made independently by McClean (56), Morrison (58), and Hirst (59). McClean found that the polysaccharide capsules of *Streptococcus pyogenes* would not appear in media to which the enzyme had been added; the capsules of group A and C strains are composed largely of hyaluronic acid, and the group B strain generates hyaluronidase as does virulent Type I pneumococcus. The enzyme from the latter organism can destroy the streptococcus capsule, but no source of hyaluronidase has been found that attacks the pneumococcus capsule. Morrison also observed the presence of hyaluronic acid in the streptococcus capsule and demonstrated its removal by the enzyme. Hirst observed that the polysaccharide capsule of group A and C, *Streptococcus hemolyticus*, could be destroyed both *in vitro* and *in vivo* by an enzyme from leech extract that may be hyaluronidase. Mice and guinea pigs were protected from intraperitoneal infection with a virulent group C streptococcus by the intraperitoneal administration of the leech extract.

Other papers dealing with hyaluronidase include a method for the biological assay of testicular diffusing factor (60), some additional evidence that decrease in viscosity and increase of reducing power of testicular digests, as well as of digests from other sources, may not be due to the same enzyme (61), the mucolytic effect of certain diffusing agents and a diazotized compound (62), the purification of the enzyme (63), and a general review of certain phases of the subject (64).

Saccharase.—In a study of Koji saccharase, Taketomi (65) reported that the enzyme has no free carbonyl group, but does contain nonreducing carbohydrate. Because the preparation employed did not give the biuret, Millon, and xanthoproteic reactions, was not destroyed by papain, pepsin, or pancreatin under the conditions used, and contained only 1.36 per cent nitrogen, Taketomi concluded that the enzyme is principally nonprotein. This conclusion is hardly justified since the low nitrogen value probably indicates the presence of nonnitrogenous impurities; the lack of saccharase destruction by proteases in a preparation containing little of the enzyme protein may

be due to some such factor as the low rate of proteolytic activity at low substrate concentration; and the failure of the preparation to elicit the protein color tests is not surprising since enzymes may produce demonstrable activity in dilutions far greater than the ranges of sensitivity of the color reactions.

An interesting investigation of the action of monomolecular films of saccharase appeared by Sobotka & Bloch (66) who found that all active films are soluble to some degree so that it is impossible to determine whether the enzyme possesses activity in the deposited state. Built-up films as thick as 45 Å retain the total enzyme activity of the film material in contrast to films of urease and other enzymes. The reduced rate in the enzymatic hydrolysis of sucrose when a foam is produced by adding egg albumin and stirring is believed by Kuzin & Bogdashevskaya (67) to result from the surface increase of the reaction mixture. One of the objections to this conclusion might be that the decrease in activity could result from adsorption of the enzyme on the egg albumin.

Other papers include a study of the influence of proteins on the reactivation of yeast saccharase (68), the conditions under which the enzyme is inactivated by ascorbic acid (69), and the application of saccharase kinetics to substantiate the biolo hypothesis of enzyme action (70).

The chief practical interest in saccharase has been its relation to the physiology of economically important plants. Harrt (71) studied the effect of a large variety of oxidizing and reducing agents, salts, and vitamins on the synthesis of sucrose from glucose and fructose in excised blades of the sugar cane plant. The necessity of aeration during synthesis was shown, and it was demonstrated that the intact tissue structure was necessary for the synthetic action. The inversion of sucrose in different parts of the sugar cane stalk as a function of the manner of storage was investigated by Lauritzen & Balch (72). Asikaga (73) claimed that no marked change in saccharase content occurs during germination of sugar cane.

Soviet investigators have reported: the synthesizing activity of saccharase is greater in the upper than in the lower leaves of pea plants (74); the removal of the budding flowers of sunflower and chrysanthemum plants results in a decrease of hydrolytic saccharase action while removal of only the leaves, or shading them, causes an increase in the action of this enzyme in the rest of the plant (75); during blossoming and at the beginning of fruiting the enzyme action

in the sunflower leaf is increased (76); the abnormal saturation with, or loss of water from, the leaves of ten-day wheat embryos produces a reduced synthesizing action of the saccharase present (77); the synthetic action of the enzyme is greater in the third and fourth than in other leaves of oat plants (78); the synthetic saccharase action increases at the expense of the hydrolytic property in buds of *Syringa vulgaris* during their development (79); and finally phosphorylation is necessary for plant synthesis of saccharose (80).

Emulsin.—A substantial advance in this field has been made recently by Pigman who investigated the relation between the structure of glycosides and their enzymatic hydrolysis by almond emulsin. He was able to show that α -mannosidase was responsible for the hydrolysis of α -phenyl-*d*-lyxoside, and that the rate of the reaction is the slowest yet reported for a phenyl glycoside (81). On the basis of available data, Pigman considers it most probable that special enzymes are required for the hydrolysis of each of sixteen fundamental hexose types (eight "*d*" and eight "*l*" isomeric aldohexopyranosides) as well as each of the α,β -isomers. The naturally occurring enzymes are expected to hydrolyze only glycosides with pyranose rings identical (except for substitutions on the fifth carbon atom) with those of naturally occurring glycosides. Confirmation of these probabilities was obtained in a subsequent study of the action of almond emulsin on synthetic heptoses and *d*-talose (82). It was also demonstrated that replacement of the oxygen bridge of β -phenyl-*d*-glycoside by sulfur reduces hydrolytic action to the point where it cannot be measured. Pigman (83) investigated the enzymatic scission of a variety of disaccharides with β -glycosidic linkages as well as a group of halogenosalicins. Introduction of halogens in the para position of salicin reduces the hydrolysis to less than one third. The relative facility of the splitting is in the order: $I > Br > Cl$.

Other studies on emulsins include purification from almonds (84), the preparation and properties of the enzymes from the snail (85), the kinetics of the enzymatic hydrolysis of the optical forms of methyl-hexyl- β -*d*-glucoside (86), a comparison of the acid and enzyme splitting of β -glucosides (87), a demonstration that neither glucose, galactose, nor gluconic acid inhibits emulsin action while these substances are effective on takadiastase (88), the enzymatic decomposition of dhurrin by the sorghum plant whose toxicity varies with the activity of the enzyme liberating hydrogen cyanide (89), and the preparation and properties of linamarase from linseed and clover (90).

Miscellaneous carbohydrases.—A number of widely diverse investigations have been reported concerning carbohydrases of certain lower forms of life and bacteria. Parkin (91) demonstrated the presence of amylase, invertase, maltase, lactase, cellulase, and hemicellulases A and B in the digestive system of the wood-boring beetle larva. Inulase, pectinase, and gelase were demonstrated by Mori & Okahuzi (92) in the internal organs of *Turbo cornutus*, and extracts of the organism were able to hydrolyze the mucilage from *Chondrus ocellatus* Holmes. Janke & Schaefer (93) observed starch hydrolysis by thirty-seven species of bacteria. Isiyama (94) reported that the gravis form of *B. diptheriae* possessed amylase and the mitis form was without activity. Leibowitz & Avinery-Shapiro (95) found that, although only gravis attacks glycogen *in vivo* and contains a phosphatase demonstrable *in vitro*, both forms break down glycogen *in vitro*. The latter authors interpret their findings as lending weight to the theory that the carbohydrate breakdown proceeds, not by hydrolysis, but by direct phosphorolysis. Epstein & Chain (96) presented data for the preparation and properties of a substrate for lysozyme consisting of a polysaccharide obtained from *Micrococcus lysodeikticus*, and Shabordin (97) developed a photometric method for the determination of the enzyme. An extensive study of the lactase of *E. coli* was made by Knopfmacher & Salle (98) in which they found that the enzyme reaction follows the Michaelis-Menten relationship, possesses a pH optimum at 7.0 to 7.5 and a temperature optimum at 46°; its activation and inactivation was also investigated. Gray & Rothchild (99) elaborated a method for the determination of melibiase activity.

ESTERASES

Lipolytic enzymes.—The relationship of ascorbic acid to lipase and esterase actions has been a subject of investigation in widely scattered laboratories. Meerzon and co-workers (100, 101) reported an inhibition of pancreatic lipase by ascorbic acid which was enhanced by the presence of copper ions, while dehydroascorbic acid had no effect; the serum and liver lipases were not appreciably influenced by ascorbic acid. A variety of reducing agents including ascorbic acid and glutathione were shown by Kayasima (102) to activate the esterase action of tissues and milk. The experiments, *in vivo*, of Kraut & Weischer (103) demonstrated that intravenous injections of 500 mg. of ascorbic acid into dogs, or several 10 mg. injections into guinea pigs, produced no particular alteration of the lipase or esterase activity of the pancreas or liver of these animals. However, Harrer & King (104), in a study of both hydrolytic and respiratory enzymes in the scorbutic guinea pig, observed that liver esterase activity decreased progressively as the vitamin was depleted to the stage of acute scurvy. No evidence was found that ascorbic acid is part of the enzyme as claimed by others. Studies of lipase and esterase in vitamin deficiencies were reported previously by Sure *et al.* (105). Other

studies on the effect of certain compounds on the activity of these enzymes have appeared by Scoz (106), who reported activation by sodium citrate and inhibition by chloral hydrate of blood and tissue tributyrinase, and by Glickstein *et al.* (107), who found an inhibition of milk lipase by both organic and inorganic iodine.

An interesting study by Schulman (108) on the orientation at the oil-water interface of esters, and their digestion by pancreatin, showed among other findings that esters whose acid and alcohol chains contain less than five carbon atoms cannot orient at interfaces and are easily digestible while longer acid or alcohol chains inhibit the hydrolysis.

The ethylbutyrate- and tributyrin-splitting capacity of the central nervous system of monkeys was mapped out by Cohn, Kaplan & Janota (109). Both substrates were hydrolyzed more actively by gray than by white matter. Weil & Jennings (24) found that the methylbutyrase action of the cells of the convoluted tubules of the rabbit kidney was greater than that of the cells of Henle's loop, and still greater than that of the cells of the collecting tubules.

Publications of a clinical nature have appeared by Meyer & Necheles (30) who found that the pancreatic lipase activity was about 20 per cent lower in a group of old people than in a younger group, by Hangleiter & Reuter (110) who observed decreased serum lipase in patients suffering from cancer, tuberculosis, liver and gall-bladder disease, and by Ehrhardt (111) who attempted to employ resistance to atoxyl inhibition of serum lipase as a test of pancreatic efficiency.

Lipase in seeds has been the subject of an investigation by Olcott & Fontaine (112) who found that dormant cottonseeds contain no lipase but that the enzyme develops during germination; it has a broad pH optimum of 7 to 8; it is activated by calcium chloride, and not affected by gossypol. In a study of lipase in hemp and pumpkin seeds, Schreiber (113) concluded that the absence of the enzyme reported by others was based on errors of method since he could demonstrate activity and determine the properties of the enzyme from these sources.

*Azolesterases.*¹—At the present time the known enzymes belonging to this group are cholinesterase, of which there may be more than one, morphinesterase, and the tropinesterases: atropinesterase, cocaines-

¹ The term azolesterase has been used by the reviewer to designate the class of enzymes that hydrolyze nitrogen-alcohol esters (114).

terase and tropacocainesterase. The differences between these members have not yet been completely established, and it is possible that certain of them may prove to be identical.

Alles & Hawes (115) pointed out that the cholinesterase of human blood serum exhibits differences compared with the enzyme of the blood cells in regard to specificity, substrate affinity, and the effects of changes in pH and salt concentration. This of course raises the possibility that the enzymes in the serum and cells are different; but the matter cannot be settled without further work. Additional studies by Glick (116) on the specificity of cholinesterase show that the splitting of *n*-acyl choline esters increases with lengthening of the hydrocarbon chain to the butyryl compound, and decreases thereafter. The effect of sodium and potassium ions on the enzyme was also considered (117).

The chief interest in cholinesterase has been attached to its possible role in the transmission of nerve impulses. The recent work of Nachmansohn and co-workers dealing with this subject must be considered more in detail since it constitutes a challenge to existing theories. From studies on the localization of cholinesterase in nerve fibers (118), employing the Linderstrøm-Lang Cartesian diver technique, and the occurrence of the enzyme in the electric organs of certain fish (119, 120), Nachmansohn and co-workers have emphasized the presence of relatively high concentrations of the enzyme at or near the surfaces of nerve cells; a parallelism between the enzyme activity and the E.M.F. developed in the electric organs was also pointed out. They have taken these findings as indicative of greater acetylcholine metabolism at or near nerve surfaces, and they believe that this supports the view that conduction along nerve fibers and across synapses differs only quantitatively. From this point further postulation is made that the chemical and electrical theories of nerve impulse transmission are compatible. This entire theoretical structure is based on the assumptions that high concentrations of cholinesterase indicate the functional presence of acetylcholine, and the acetylcholine is somehow responsible for nerve transmission along the fibers. In support of the first assumption, the claim was made that cholinesterase is a specific enzyme (119) (Easson & Stedman, and Glick were cited), and its presence precludes that of its specific substrate, acetylcholine. Unfortunately the papers cited indicate rather that the enzyme is far from specific for just acetylcholine, being able to hydrolyze quite a variety of esters. The danger of reasoning that the presence of a

high concentration of an enzyme allows one to assume the presence of a particular substrate is apparent when it is recalled that certain rabbits possess high concentrations of enzymes capable of splitting atropine, heroin, etc.; one can hardly assume these alkaloids to be normally present in the animals. Even if these difficulties did not exist and the presence of acetylcholine itself had been established, there would still be the burden of demonstrating just how this ester can be responsible for the transmission of an impulse along the length of a nerve fiber, a problem that these investigators themselves have acknowledged (120). The present criticism is not that the relatively high concentration of the enzyme is without significance, but that the extensive theoretical structure developed rests on an experimental foundation too meagre to justify it.

A great variety of other investigations involving the role of cholinesterase in the nervous system have appeared (121 to 128). The question of whether the enzyme itself is liberated on neural stimulation has been considered by Runcan (129), who claimed that stimulation of the ischiac nerve resulted in an increase of the cholinesterase in the gastrocnemius muscle of the dog, while the enzyme in the blood from the leg decreased. Trowbridge (130) observed no increase in enzyme activity in the venous blood from the leg muscles of the frog and cat when they were stimulated.

Interest has centered on the relationship of thiamin to cholinesterase since it has been shown that thiamin inhibits the enzyme both *in vivo* and *in vitro* and potentiates the physiological action of acetylcholine. Brücke & Sarkander (131) demonstrated that thiamin sensitizes leech muscle to acetylcholine and confirmed (132) the earlier finding of Antopol, Glaubach & Glick (133) that the cholinesterase level in the blood serum of beriberi pigeons is abnormally elevated. Pighini (134) reported that the enzyme activity of sciatic nerve suspensions from pigeons fell during thiamin deficiency, while Zeller & Birkhäuser (135) observed a decrease in the liver, but no change in the brain enzyme in thiamin-deficient rats. In line with earlier studies from the laboratories of Minz and Abderhalden, Brecht (136) found intensification by thiamin of the vasoconstricting effects of acetylcholine in the frog; Nitzescu & Teodoru (137) observed the same in the frog, leech, and dog; Dick & Hege (138) noted increased peristaltic action upon administration of thiamin to rats deficient in the vitamin; and Byer & Harpuder (139) observed thiamin sensitization of the action of acetylcholine on rat intestine. The last mentioned investiga-

tors found thiamin to be inactive on frog heart and rectus muscle, rabbit intestine, and denervated cat ear, from which they concluded that the vitamin has no apparent effect upon cholinesterase in tissues. (This conclusion may be true in the cases and under the conditions employed by the authors, but the evidence that it is not so in other instances should be borne in mind).

Studies on the action of certain drugs on cholinesterase have led many observers to conclude that the pharmacological effects of these compounds, at least to some degree, can be accounted for by their effects on the enzyme. Thus Slaughter & Lackey (140) found that injection of morphine into dogs resulted in decreased activity of the serum cholinesterase and interpreted this as another indication of a cholinergic mechanism for morphine action; they failed to observe inhibition *in vitro*. However, Eadie (141) demonstrated the competitive inhibition of cholinesterase by morphine *in vitro* and studied the effects of pH on the inhibition.

Rosen & Borenstein (142) claimed that the pharmacological effect of β -erythroidine results from a release of cholinesterase while prostigmine overcomes erythroidine action by inhibiting the enzyme, but no experimental data were offered or quoted in support of this odd theory. Harris & Harris (143) showed that β -erythroidine had no effect *in vitro* on the enzyme in human serum, while curare was a strong inhibitor even though both of these drugs produce a similar muscle paralysis. In another study the latter authors (144) demonstrated that intravenous injection of curare also inhibited the enzyme in serum. Apparently the role of cholinesterase, if any, in the action of these drugs is too obscure to permit a positive point of view.

The inhibitory action of prostigmine on cholinesterase has been used by Mendez & Ravin (145) to explain certain phases of the action of the drug on the circulatory system. These authors took cognizance of the previously known fact that prostigmine also has a direct action independent of its effect on the enzyme. Thompson & Tice (146) reported that the pharmacological effect of prostigmine follows more closely the depression in potassium concentration than that of cholinesterase in the serum.

The interesting finding that methylene blue, both *in vitro* and *in vivo*, inhibited the enzyme to the same high degree as physostigmine was made by Rentz (147). However, since the dye did not have the pharmacological properties of physostigmine, Rentz concluded that the latter does not act through its inhibition of the enzyme. This de-

duction is not really justified from the available data. In a study of the effect of various dyes on cholinesterase activity, Massart & Dufait (148) reported that acid dyestuffs such as Chicago blue and Congo red have no effect, while alkali dyes inhibit the enzyme, and the presence of a quaternary ammonium radical increases this inhibition in proportion to the degree of its dissociation.

The *in vitro* experiments of Adriani & Rovenstine (149) showed that none of the common volatile anesthetics affected cholinesterase activity, while certain local anesthetics inhibited the enzyme. Ettlinger, Brown & Megill (150) reported that the potentiation of acetylcholine action by alcohol and ether depends, at least in part, on their inhibitory action on cholinesterase. Heim & Fahr (151) found that lower concentrations of urea, urethane, sodium barbital, and isoamyl and ethyl alcohols activated the enzyme, while higher concentrations inhibited it. The effects of cardio- and vascular-active drugs on cholinesterase were studied by Zinnitz & Rentz (152).

Further studies of this enzyme in pathological conditions of humans have been made in the hope of obtaining criteria for possible new diagnostic tests. Hawes & Alles (153) surveyed a good deal of the work published in this field and concluded "there is today no condition known for which the assay of blood cholinesterase can be expected to give individual results of consistent diagnostic value." Recent results which are in accord with this conclusion indicate that the enzyme level is independent of allergic states (154), cannot be considered of diagnostic value in anemias (155), and bears no relation to the clinical state of schizophrenics (156). However, the diagnostic possibilities in liver disease and hyperthyroidism would appear promising from earlier work of Antopol, Schiffrin, & Tuchman which has been recently confirmed and extended in the case of liver disease by McArdle (157). The latter investigated the enzyme test as a means of differentiating jaundices of hepatic and obstructive origins, but the distinction cannot be considered satisfactorily established as yet. Other studies on cholinesterase include the effects of age, sex, and sex hormones (158 to 161), changes during human fetal development (162), and the enzyme in lymph (163) and in snake venom (164). Two reviews dealing with this enzyme have appeared (165, 166).

The hypothesis that cholinesterase and the enzyme hydrolyzing acetylthiamin are the same has been advanced by Massart & Dufait (167) and Reichert (168). The former authors based their conclu-

sion on similarities in kinetic behavior, inhibition by agents such as arsenite, fluoride and physostigmine, and activation by calcium ion; the latter author resorted to parallels in physiological experiments. None of these criteria can be considered real tests of identity.

Evidence was presented for the possible existence of atropinesterase, cocainesterase and tropacocainesterase as distinct enzymes by Glick & Glaubach (169). They observed no relation between the occurrence of atropinesterase in rabbits and season, sex, color, age, or weight; it was shown that the absence of the enzyme from certain animals was not likely the effect of a naturally occurring inhibitor.

Wright (170) made the interesting observation that the blood sera of certain rabbits were capable of enzymatically hydrolyzing both acetyl groups from diacetylmorphine, the single acetyl group from monoacetylmorphine and the phenolic acetyl from diacetyldihydromorphine, while the sera of other animals affected only the phenolic acetyl in diacetylmorphine, did not deacetylate monoacetylmorphine, and only slowly split the phenolic acetyl from diacetyldihydromorphine. Neither group of sera hydrolyzed the alcoholic acetyl from monoacetyldihydromorphine. These findings remind one of the two classes of rabbit sera with respect to atropine hydrolysis mentioned above.

Phosphatases.—The properties of several of the less common phosphatases have been recently studied. Thus Sjöberg (171) investigated the blood pyrophosphatase which occurs chiefly in the red cells; plasma contains a strong inhibitor for the enzyme. The enzymatic hydrolysis of choline β -glycerophosphate by snake venom and extracts of rice husks was demonstrated by Ravazzoni & Fenaroli (172). An interesting study of the mode of action of "ribonuclease" was made by Eiler & Allen (173) who reported that this enzyme appears to be a specific phosphatase liberating a group characteristic of a secondary phosphate dissociation, and from studies with substrates of known structure, they concluded that the enzyme is not a phosphomonoesterase, a phosphodiesterase, or a phosphoamidase. Schmidt & Levene (174) had previously maintained the view that the enzyme is a depolymerase which breaks down high molecular weight tetranucleotides to those of lower molecular weight. Later Allen & Eiler (175) used the crystalline pancreatic enzyme to elucidate the structure of ribonucleic acid. In a similar manner Schramm (176) employed enzymatic action to study the constitution of the nucleic acid from tobacco mosaic virus. Travia & Veronese (177) used alkaline phos-

phatase to learn something of the phosphoric acid structure of casein. The hydrolysis *in vivo* of tocopherol phosphoric acid ester was demonstrated by Engel (178), and the phytase in plasma and erythrocytes of various species of vertebrates was studied by Rapoport *et al.* (179). A yeast enzyme, which is probably identical with "top yeast phosphatase," has been investigated by Westenbrink *et al.* (180), who found that the enzyme hydrolyzes thiamin pyrophosphate by means of the intermediate formation of the orthophosphate; a pH optimum at 3.7 was observed.

A study of the influence of various agents on phosphatases has been approached from diverse angles. Albers (181) showed that both ultraviolet and x-rays rapidly destroy purified horse kidney phosphatase while the rate of destruction of the less pure bile enzyme is slower. Activation of phosphatases by cations has been extensively investigated (182 to 185), and inhibition by certain anions (185) and thiamin (186) has also been studied.

Purification work has been carried out on alkaline phosphatase by Albers (187) using horse kidney as the source, and by Marsal (188) who employed pig kidneys. Other papers have appeared on the occurrence and properties of phosphatase (189 to 191).

A highly ingenious technique that promises to be of great value was developed simultaneously and independently by Gomori (192) and Takamatsu (193) to study the histological distribution of alkaline phosphatase. Both investigators exploited the fact that the tissue enzyme survives alcohol fixation and subsequent paraffine or celloidin embedding. When the fixed sections are immersed in a buffered substrate solution containing calcium ions, calcium phosphate precipitates at sites of phosphatase activity, and a calcium stain may be then applied. Acid phosphatase cannot be brought out by the procedure because of the solubility of calcium phosphate in the lower pH range. Hence for this enzyme Gomori (194) used a lead phosphate precipitate which could be converted to the sulfide. The methods have recently been applied to studies on the distribution of the enzyme in normal and pathological tissues (194 to 198). Hilde & Marron (199) employed the technique for both plant and animal tissues; germinating wheat seeds were studied particularly.

Clinical investigations on phosphatases have been carried out on a wide variety of syndromes. In the last few years special attention has been given, particularly by Gutman and co-workers, to the serum acid phosphatase level in relation to bone metastases from carcinoma

of the prostate. The abnormally high activities obtained follow the severity of the condition and hence are of diagnostic and prognostic value. The prostate is unusually rich in acid phosphatase and is chiefly responsible for the appearance of this enzyme in semen (200, 201), and the enzyme also occurs in high concentration in the bone metastases from prostatic cancer. Huggins *et al.* studied the effect on the enzyme of castration (197 to 202) and hormone (202) treatment of the disease, and reported decreased activity as the result of both castration and estrogen therapy, while androgens produced an increase. Herger & Sauer (203) and Müller (204) investigated the clinical significance of serum acid phosphatase in this disease, and Gutman (205) pointed out the effect of hemolysis on the determination of the serum enzyme in the course of a study on the erythrocyte phosphatase. The enzyme in red cells was also investigated by Tanigawa (206).

Papers also appeared that deal with serum phosphatase in infected bone fractures (207), Paget's disease (208), rickets (209), jaundice (210, 211), hypothyroidism (212), infantile scurvy (213), bone tumors (214), dental tartar formation (215), and even in normal individuals (216). In addition the enzyme was studied in liver in cases of cancer (217) and von Gierke's disease (218), in early callus (219), and in the elementary bodies of vaccinia (220).

Additional studies of a diverse nature include phosphatase in chick perosis (221), in development of the sardine skeleton (222), in molluscs (223), in fish sperm (224), in bovine male sex glands (225), in the various phases of the life of the silk worm (226), in the serum of cattle and sheep (227), in rat feces (228), in rat plasma during embryonic and tumor growth (229), in the embryonic tissue fluid of the chicken (230), in the proximal convoluted tubules in relation to phlorhizin glycosuria (231), in rat femurs after treatment with adrenal cortical substances and parathyroid extract (232) and thyroxine and thymus extract (233), in rat skeleton after administration of estradiol benzoate (234), in yeast (235), and in the course of the withering of the leaf blades in wheat sprouts (236).

Miscellaneous esterases.—Cholesterol esterase, in relation to resorption and transport of fat, was studied by Schramm & Wolff (237) who also reported on the enzymatic esterification of steroids (238). Sperry & Brand (239) investigated the cholesterol esterase in liver, brain, and blood in regard to the hypothesis supported by Schramm & Wolff that fatty acids are transported as cholesterol esters, but no

conclusive findings were obtained. Rozenfel'd & Rukhel'man (240) reported evidence for the presence of an aldehyde group in sulfatase which they interpret as a component of the prosthetic group ("agon") of the enzyme through which it is bound to the colloidal part ("pheron").

OTHER ENZYMES

Evidence for the existence of enzymes that are as yet poorly defined has been presented by Kopac (241) who found an "enzyme" that disintegrates the fertilization membrane of *Arabacia*, by Abraham & Chain (242) who observed the enzymatic destruction of penicillin by extracts of *B. coli*, and by Greengard, Stein & Ivy who reported the enzymatic inactivation of secretin (243) and cholecystokinin (244) by dog serum.

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THE CHEMISTRY OF THE ACYCLIC CONSTITUENTS OF NATURAL FATS AND OILS¹

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Some reduction in the number of investigations reported from European centres of research is naturally observable in the biennial period under review but, with the less restricted output from America, where special activity in this field has been prominent for some years, the total volume is nevertheless considerable. It has become increasingly evident that the general picture of the chemistry of natural fats discloses on the one hand wide similarities both in the nature of the combined fatty acids and of the resulting glycerides, and on the other hand notable divergencies from the types of structure more usually encountered. Thus the chief component acids of fats are comparatively few in number—oleic, linoleic, and palmitic acids being the most prominent, and the other more usual saturated or unsaturated members being closely related to these in constitution; however, some species, especially in the vegetable kingdom, give rise to quite exceptional types of unsaturation, cyclic structure, or even branched instead of normal carbon chains. Similarly, although the structure of natural triglycerides is overwhelmingly characterised by a tendency to indiscriminate or "even" distribution of the different fatty acids throughout the triglyceride molecules, some well-defined exceptions (or, as it now appears, modifications) of this fundamental rule are discernible here and there. Accordingly, recent progress is here treated so as to emphasise, as far as may be, both these aspects of the chemistry of natural fats. The subject is conveniently subdivided broadly into (a) matters dealing with individual fatty acids, (b) component acids reported in various groups of natural fats, and (c) questions connected with their glyceride structure.

INDIVIDUAL FATTY ACIDS

The use of lead tetra-acetate in acetic acid solution for the transformation of hydroxylated fatty acids has received considerable recent attention. Mendel & Coops (1) have utilised this mode of oxidation

¹ The literature available up to October, 1941, has been examined.

in degrading a higher fatty acid into its next lower homologue; e.g., stearic acid is successively converted into α -bromo- and α -hydroxystearic acid, the latter being oxidised by the lead tetra-acetate solution into heptadecanal which, by passage of air through the tetra-acetate solution, passes into *n*-heptadecoic acid. Hsing & Chang (2) employed the same agent to obtain 85 to 90 per cent yields of *n*-nonaldehyde and the semi-aldehyde of azelaic acid from 9,10-dihydroxystearic acid. The method is also given by Scanlan & Swern (3), who have effected an improvement in the technique of hydrogenation (4) of oleic acid by hydrogen peroxide and acetic acid. Scanlan & Swern state that the scission into aldehydes is better effected by subsequent addition of red lead to the acetic acid solution of dihydroxy-fatty acid (without isolating the latter). Hilditch & Jaspersen (5) have noted that the rates of oxidation by lead tetra-acetate of the lower melting form of the 9,10-dihydroxystearic acids (m.p. 95°), and of those of the 9,10,12,13-tetrahydroxystearic acids (m.p. 144° and 136°) are in each case far more rapid than those of the higher melting isomers (m.p., resp., 132° ; and 163° and 172°). King (6) found that good yields of non-aldehyde and azelaic semi-aldehyde result from the action of aqueous periodic acid on 9,10-dihydroxystearic acid.

The isolation of oleic, linoleic, and linolenic acids in comparatively high states of purity by crystallisation from acetone at temperatures down to -50° , and evidence that all three natural acids were exclusively *cis* forms, was fully discussed in the previous review (7). Brown *et al.* (8, 9) have since used low-temperature crystallisation in the preparation of pure ricinoleic acid (m.p. 5.5°) from castor oil acids and of arachidonic acid (95 per cent pure) from ox adrenal phosphatides. Stewart & Wheeler (10) have examined the melting point curves, and defined the eutectic mixtures present, for binary mixtures of oleic, linoleic and linolenic acids. From the pure acids, Wheeler *et al.* (11) synthesised by direct esterification triolein (obtained in three polymorphic forms, m.p. -32° , -12° and 5°) and trilinolein (obtained in two polymorphic forms, m.p. -43° and -13°). The infra-red and Raman spectra of oleic, linoleic, linolenic, elaidic, and lin-elaidic acids have been observed by McCutcheon *et al.* (12), the results conclusively supporting the view that the natural acids contain only *cis* linkings, whilst the isomerised (elaidic) acids are wholly *trans* compounds. The production of isomeric linoleic and linolenic acids by addition of bromine (or during subsequent debromination) is still a matter of discussion. Kass *et al.* (13) prepared elaidolinolenic acid,

m.p. 29° – 30° , which gave a solid hexabromostearic acid, m.p. 169° – 170° , in 31 per cent yield, the rest being ether-soluble liquids. Debromination of both solid and liquid bromo-compounds occurred without isomerisation, confirming the previous conclusions of Kass & Burr that a polythenoid acid may form several "bromides" and that no significant isomerisation takes place on debromination. On the other hand, Matthews *et al.* (14) have succeeded in separating linoleic acid, prepared by debromination of the crystalline tetrabromostearic acid, by low-temperature crystallisation into the pure " α "-acid (m.p. -5.2° to -5.0°) and other fractions of linoleic acid which gave lower yields of solid tetrabromostearic acid on addition of bromine. Similar, but less conclusive, results were obtained with linolenic acid from solid hexabromostearic acid, the solubilities of the isomeric acids in this case being too close to permit good separation. Consequently these data reinforce the view of Brown & Frankel (15) that acids prepared by debromination contain appreciable amounts of isomeric acids which, it is believed, are the result of isomerisation during the removal of bromine. Frankel & Brown (16) have improved the low-temperature crystallisation of linoleic acid from petrol at -60° , and obtained from corn oil a specimen of linoleic acid identical in properties with that obtained by repeated crystallisation of the acid obtained by debromination (14).

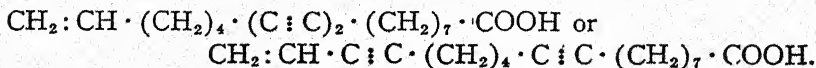
Moore (17, 18) observed some years ago that, whilst natural linoleic and linolenic acids show only general absorption in the ultraviolet, the products obtained from these acids after prolonged heating with caustic alkali solutions give well-marked absorption bands, and that the $\Delta^{9,12,15}$ -triethenoid system of linolenic acid, for example, was rearranged to a conjugated ($\Delta^{10,12,14}$ -) system. Kass *et al.* (19) have confirmed this, and have also shown that reduction of methyl linoleate with sodium and alcohol yields a mixture of $\Delta^{9,12}$ - and $\Delta^{10,12}$ -octadecadienols. Houston *et al.* (20) found that the C_{18} unsaturated acids of cow milk fat, in contrast to those of pasture grasses, showed definite absorption in the ultraviolet. Bradley & Richardson (21) have pointed out that the well-defined absorption bands at about 230 m μ for conjugated $\Delta^{9,11}$ - or $\Delta^{10,12}$ -octadecadienoic acids and at about 270 m μ for conjugated $\Delta^{9,11,13}$ - or $\Delta^{10,12,14}$ -octadecatrienoic acids are of great use in judging, from their intensity, the proportions of such conjugated acids when present in a fatty oil. They have confirmed the earlier observations of Steger & van Loon (22) that heat treatment of such oils as linseed, or the methyl esters of linoleic or linolenic acids, causes

rearrangement of the unsaturated systems to conjugated forms, which on further heating undergo polymerisation to dimeric and trimeric forms (23, 24). Studies of this kind are mainly concerned, of course, with the changes which occur when a "drying" oil, with or without prior heat treatment, is converted into a paint or varnish film, but the underlying chemical processes, which are becoming clearer as the result of work by many investigators, merit notice in a review of the chemistry of the fats. It has also been shown that the octadecadienoic glycerides produced when castor oil is dehydrated consist in part of the (conjugated) $\Delta^{9,11}$ -acid, together with a good deal of the $\Delta^{9,12}$ -acid (21, 25, 26).

Arachidonic acid, when submitted to the action of ozone by Shinowara & Brown (9), yielded products which suggested tentatively that it might be a $\Delta^{6,10,14,18}$ -*n*-eicosatetraenoic acid; on the other hand, the acids isolated after oxidising arachidonic acid with aqueous alkaline permanganate led Dolby *et al.* (27) to propose $\Delta^{5,8,11,14}$ -*n*-eicosatetraenoic acid as a possible structure.

Some rare vegetable unsaturated fatty acids.— Δ^9 -Tetradecenoic (myristoleic) acid is a well-recognised minor component of most fats of aquatic origin and also of cow milk fats and some animal depot fats, but has not appeared in any vegetable fat hitherto. Atherton & Meara (28), however, have found it to be a major component of the seed fat of *Pycnanthus Kombo*, where it forms 24 per cent of the combined fatty acids; as is usual in seed fats of the Myristicaceae, the other major component is myristic acid (62 per cent).

Unusual members of the C_{18} group of unsaturated acids continue to crop up. In the seed fat of *Ongokea Gore* Steger & van Loon (29) discovered an acid which contains one ethenoid and one acetylenic linking, the compound being $\Delta^{9,9}$ -octadecinenoic acid. In another species, *O. klaineana*, Castille (30) has found the seed fat to contain an acid $C_{18}H_{28}O_2$ which possesses one ethenoid and two ethinoid groups. It yields on ozonolysis oxalic and adipic acids, ethyl hydrogen azelate and formaldehyde, the latter proving that the double bond is at the remote end of the carbon chain. The occurrence of terminal ethenoid unsaturation, as also of two acetylenic linkings, in a natural higher fatty acid is unique. The structure assigned is octadec- Δ^{17} -en- $\Delta^{9,11}$ (or 15)-di-inoic acid,



The seed oil of *Sterculia foetida*² (Java olive) has long been known to undergo sudden exothermal polymerisation when heated at 250°. Hilditch *et al.* (31) have found that this is due to glycerides of a peculiar unsaturated acid which forms about 70 per cent of the total fatty acids and has the composition $C_{19}H_{34}O_2$. It contains a branched chain and two double bonds, which are probably, but not certainly, conjugated; one ethenoid linking is in the $\Delta^{11:12}$ - position, but the location of the other has not been definitely ascertained. The compound is, however, possibly a 12-methyl- $\Delta^{9,11}$ -octadecadienoic acid, $CH_3 \cdot (CH_2)_5 \cdot C(CH_3) : CH : CH : CH : (CH_2)_7 \cdot COOH$. The seed fats of *Sterculia* species appear unusually diverse in character, since Ueno & Ueda (33) found that of *S. platanifolia* to contain only the common acids palmitic and stearic (25 per cent), oleic (46 per cent) and linoleic (29 per cent).

Puntambekar & Krishna (34) found in 1937 that the seed fat of *Ximenia americana* contained a *n*-hexacosenoic acid $C_{26}H_{50}O_2$; this has since been confirmed by Boekennoogen (35), who has also noted the presence of small quantities of an acid, $C_{30}H_{58}O_2$. He states the percentage distribution of the component acids to be stearic, 4; *n*-hexacosic, 2; oleic, 54; linoleic, 10; *n*-hexacosenoic, 25; and *n*-tricosenoic, 5. The structures of the two higher unsaturated acids are respectively those of Δ^{17} -hexacosenoic and Δ^{21} -tricosenoic acids. Boekennoogen has drawn attention to the existence of natural seed fat acids of the general formula $CH_3 \cdot (CH_2)_7 \cdot CH : CH \cdot (CH_2)_x \cdot COOH$, where *x* is 3, 7, 11, 15, or 19, in contrast to the more widely distributed group (especially in aquatic and animal fats) of the general formula $CH_3 \cdot (CH_2)_x \cdot CH : CH \cdot (CH_2)_7 \cdot COOH$ (where *x* may be 1, 3, 5, 7, or 9).

COMPONENT ACIDS OF NATURAL FATS

Analysis of fatty acids by ester fractionation, and new forms of apparatus for this purpose, have formed the subject of further reports. Weitkamp & Brunstrum (36) described a new form of fractionating column which gives sharp separations of the homologous esters, the boiling point curves showing flat plateaux with sharp transitions. Saponification equivalent determinations can be eliminated when this apparatus is employed.

² The composition of the fatty acids of the seed and fruit coat fats previously ascribed by Hilditch & Stainsby (32) to *S. foetida* really refer, owing to an error in botanical identification, to those of *Dacryodes rostrata* Lam (syn. *Canarium Kadondon*, Bennett).

The effect of prolonged vacuum distillation of polyethenoid esters from codliver oil on the constitution of the highly unsaturated esters has been examined by Norris *et al.* (37) by means of absorption spectra, iodine values and saponification equivalents. They found that little isomerisation took place during fairly prolonged fractional distillation; the isomerisation was insufficient to interfere with the composition and structure of the distilled esters, but isomerised material concentrated in the undistilled residues.

VEGETABLE FATS

An exhaustive search for acids of the C_{2n-1} as well as of the C_{2n} series in coconut oil was made by Jantzen & Witgert (38), starting with 25 kg. of oil and eventually obtaining, by progressive and systematic ester fractionation at low pressures, fractions ranging from 12 gm. to 0.1 gm. of individual esters. At least 99.5 per cent of the acids were of the even-numbered (C_6 to C_{18}) series; only about 0.1 per cent of odd-numbered acids (C_9 , C_{11} , and C_{13}) was detected, these probably due to oxidation and not synthesised in the original fat.

The acids of coconut oils from Hainan, South China (39) and from the inner and outer South Sea Islands (40) have been determined by Nobori *et al.*, who found the following respective percentage distributions for specimens from the Chinese and South Sea Islands: octoic, 8.7, 9.2; decoic, 8.1, 9.7; lauric, 51.3, 44.1; myristic, 13.1, 15.9; palmitic, 7.5, 9.6; stearic, 2.0, 3.2; oleic, 5.5, 6.3; linoleic, 2.3, 1.5. These data, and also those for babassu fat (41), fall close to those given by previous workers, the babassu fatty acids being recorded as octoic, 4.1; decoic, 7.6; lauric, 45.1; myristic, 16.5; palmitic, 5.8; stearic, 5.5; arachidic, 0.7; oleic, 11.9; and linoleic, 2.8 per cent. Jamieson & Rose (42) have found the percentage distribution of the acids in the kernel fat of the Uruguayan *Cocos pulposa* to be hexoic, 1.5; octoic, 9.4; decoic, 13.2; lauric, 34.4; myristic, 6.6; palmitic, 1.8; stearic, 1.3; oleic, 22.4; and linoleic, 2.4 per cent.

The chief component acids of virola fat (from *V. surinamensis*) were found (28) to be lauric (14.9 per cent), myristic (73.2 per cent), palmitic (5.0 per cent), and oleic (6.4 per cent). Those of the seed fats of *Salvadora oleoides* and *S. persica* were determined (43) respectively as decoic (1.5, 1.0 per cent), lauric (21.2, 19.6 per cent), myristic (52.9, 54.5 per cent), palmitic (18.9, 19.5 per cent), and oleic (5.5, 5.4 per cent).

The seed fat of *Canarium commune* ("Java almond") was observed by Steger & van Loon (44) to contain as component acids palmitic (29.0 per cent), stearic (9.7 per cent), oleic (38.3 per cent), linoleic (21.8 per cent), and linolenic (1.2 per cent). It does not polymerise on heating, whereas that from the related *Dacryodes rostrata* (syn. *C. Kadondon*, Bennett) exhibits exothermal polymerisation at 250°, due to a resin accompanying the fat (32). The seed fat of *D. rostrata* also differs materially from that of *C. commune* in its component acids, which were found, for two different specimens of the seed fat (32, 31) to be respectively myristic (0.0, 1.0 per cent), palmitic (10.7, 12.7 per cent), stearic (40.3, 30.9 per cent), arachidic (2.1, 3.1 per cent), oleic (43.6, 49.5 per cent), and linoleic (3.3, 2.8 per cent).

Soybean oils may vary widely in iodine value (101 to 151) from the usual figure of about 130. Dollear *et al.* (45) showed that the saturated (palmitic and stearic) acid content remains uniform, the oils of higher iodine value containing much less oleic and much more linoleic (and linolenic) acids. Their data for a normal oil (iodine value 131.6) and for oils of very low (101.6) and very high (151.4) iodine value were respectively: saturated acids (12.7, 12, 13.5 per cent), oleic (27.7, 60, 11.5 per cent), linoleic (53.7, 25, 63.1 per cent), and linolenic (5.9, 2.9, 12.1 per cent).

The component acids of stillingin seed oil (*S. sebifera*, syn. *Sapium sebiferum*) from United States and Chinese crops were determined by Jamieson & McKinney (46) to be respectively: palmitic (4.4, 5.9 per cent), stearic (1.4, 2.6 per cent), arachidic (0.3, 0.1 per cent), oleic (7.7, 10.4 per cent), linoleic (56.3, 49.9 per cent), and linolenic (24.6, 25.4 per cent).

A large number of other seed fats whose component acids are confined to the common type of saturated (palmitic and stearic), oleic, and linoleic (and sometimes linolenic) have been reported in detail. Of seed fats which contain over 10 per cent of saturated acids (the rest being oleic and linoleic in varying proportions) the following may be mentioned: neem oil [palmitic (14.9 per cent), stearic (14.4 per cent), oleic (61.9 per cent), linoleic (7.5 per cent)] (47); Indian shaddock [palmitic (20.7 per cent), stearic (15.3 per cent), oleic (55.5 per cent), linoleic (8.1 per cent)] (48), *Sideoxylon ferrugineum* [saturated acid, mainly palmitic (29.4 per cent), oleic (14.2 per cent), linoleic (56.4 per cent)] (49). In the following instances appreciable proportions of linolenic acid were noted: lallemantia [sat-

urated acids (12.2 per cent), oleic (7.5 per cent), linoleic (25.0 per cent), linolenic (55.3 per cent)] (50); fenugreek [saturated acids (10.6 per cent), oleic (21 per cent), linoleic (37 per cent), linolenic (19 per cent)] (51); wild rose [saturated acids (4.5 per cent), oleic (29.3 per cent), linoleic (56.7 per cent), linolenic (9.5 per cent)] (52). In most of the seed fats recorded, however, which contain only minor quantities of saturated acids, 75 per cent or more of the acids consist of oleic and linoleic acids in varying proportions; in this large group data have recently been given for the following seed oils: myrobalan (*Terminalia chebula*) (53), *Bauhinia variegata* (54), *Euphorbia lathyris* (55), *Sapindus drummondii* (56), *Schizandra chinensis* (57), *Xanthium strumarium* (58), *Martynia diandra* (59), *Carya cordifolia* (60), *Setaria itarica* (61), *Viburnum opulus* (62) and *Capparis spinosa* (63).

In the group of chaulmoogra and related oils, small quantities of cyclopentene acids which are lower homologues of hydnocarpic and chaulmoogric acids have been found by Cole & Cardoso (64), who have termed them aleprolic ($C_6H_8O_2$), aleprestic ($C_{10}H_{16}O_2$), alepylic ($C_{12}H_{20}O_2$, m.p. 32°), and alepric ($C_{14}H_{24}O_2$, m.p. 48°) acids. The component acids of five typical oils of this group have now been determined by these investigators (65), who separated the mixed fatty acids of each oil into solid and liquid portions by crystallisation from 80 per cent alcohol, and submitted the esters of each group to fractional distillation; their data are summarised in Table I.

TABLE I
COMPONENT ACIDS OF OILS OF THE CHAULMOOGRA GROUP

Component acids	<i>Carpotroche brasiliensis</i> , Sapo-cainha oil	<i>Oncoba echinata</i> , Gorli oil	<i>Hydnocarpus Wightiana</i> , Maratti oil	<i>Hydnocarpus anthelemtica</i> , Lukrabo oil	<i>Taraktogenos Kurzii</i> , Chaulmoogra oil
	%	%	%	%	%
Palmitic acid.....	6.6	7.8	1.8	7.5	4.0
Oleic acid.....	6.3	2.2	6.5	12.3	14.6
Lower homologues of hydnocarpic acid.....	?	?	3.4	0.1	0.4
Hydnocarpic acid ($C_{16}H_{32}O_2$)...	45.0	nil	48.7	67.8	34.9
Chaulmoogric acid ($C_{18}H_{32}O_2$)..	24.4	74.9	27.0	8.7	22.5
Gorlic acid ($C_{18}H_{30}O_2$).....	15.4	14.7	12.2	1.4	22.6

The fatty matter in the leaves of a number of land plants and trees was examined by Tsujimoto (66) in regard to the content of highly unsaturated acids present; small proportions of linolenic acid were observed in clover and in the leaves of all the other species studied except pine, but highly unsaturated acids of the C_{20} and C_{22} series, present in algae and characteristic of fats from aquatic sources, were not detected in any instance. Tang & Hso (67) found lauric, oleic, linoleic, and linolenic acids in the leaf fat of *Leonurus sibiricus*.

MARINE ANIMAL FATS

A comprehensive monograph (68) on marine animal oils with particular reference to those of Canada has been published by the Fisheries Research Board of Canada and is edited by Dr. H. N. Brocklesby. It contains a full account of Canadian and other marine animal fats, including the most recent investigations in this field. The composition of the depot fats of the New Zealand ling has been given by Shorland (69); there was no significant seasonal variation in the fatty acid composition of the ling liver oils, which were of the usual "marine" type except for somewhat larger amounts than usual of unsaturated C_{18} acids. Eels were fed by Lovern (70) on individual fatty acid esters but the changes resulting in the depot fats were too small to permit of definite conclusions being reached; however, ingestion of ethyl myristate caused an increase in tetradecenoic acid, while ethyl palmitate effected an increase in hexadecenoic acid; mixed unsaturated esters gave results suggesting hydrogenation of hexadecenoic to palmitic acid. Nobori (71), working with about a ton of mixed fatty acids from herring oil, obtained evidence of the presence therein of octoic (0.9 per cent), decoic (0.9 per cent), and lauric (2.2 per cent) acids.

The fat of the turtle *Chelone mydas* was found by Ogata & Minato (72) to contain dipalmitostearin and myristodipalmitin; the percentage distribution of its total component acids was hexoic, 3.5 (—); lauric, 14.2 (13.3); myristic, 7.2 (10.6); palmitic, 15.2 (17.0); stearic, 6.8 (4.1); tetradecenoic, 2.6 (1.3); hexadecenoic, 10.9 (7.8); oleic, 39.4 (39.6), with a small amount of highly unsaturated acids (6.1). These data accord with those of an earlier analysis by Green & Hilditch (73) (the figures for which are given in parentheses). The body fat of the land crab, *Birgus latro*, contains a still more unusual variety of saturated acids. Hilditch & Murti (74) found the component acids to be octoic (1.5 per cent), decoic (5.3 per cent),

lauric (47.5 per cent), myristic (19.0 per cent), palmitic (13.1 per cent), stearic (1.7 per cent), tetradecenoic (0.7 per cent), hexadecenoic (2.2 per cent), oleic (5.3 per cent), linoleic (1.5 per cent), and unsaturated C_{20-22} acid (2.2 per cent). The fat consisted of 66 per cent of fully saturated glycerides [lauric (54.8 per cent), and myristic (20.5 per cent)]. In this instance the peculiar composition of the fat, for an animal, is probably due to the feeding habits of the crab. It lives largely on coconuts, so that its body fat is composed mainly of assimilated coconut fat with only a subordinate proportion of the glycerides typical of the fat of an aquatic animal.

LAND ANIMAL FATS

The lipids of the earthworm were studied by Lovern (75) who found that about 60 per cent of the total fatty matter is insoluble in acetone (phospholipids and waxes). The acetone-soluble fraction contained much sterol esters but little triglycerides. In addition to fatty acids of the usual range (C_{10} to C_{22}) and higher (wax) fatty acids some unidentified acids were present. The typical glyceride and phospholipid acids were marked by a very low content of hexadecenoic acid and high proportions of stearic acid in relation to the palmitic acid present.

The component acids of bone marrow fat from oxen were found by Hilditch & Murti (76) to resemble closely those of typical ox depot fat of the softer (rump) types: myristic (2.6 per cent), palmitic (32.3 per cent), stearic (15.5 per cent), hexadecenoic (3.0 per cent), oleic (43.2 per cent), and linoleic (2.6 per cent).

Some additional facts have been published with regard to depot fats of pigs, sheep, and oxen. Prolonged starvation of pigs causes relatively slight alterations in the fatty acid compositions of the perinephric fats and of those of the back even when over 70 per cent of the fats have been mobilised (77). In the earlier stages of fasting, there is relatively less mobilisation of linoleic and unsaturated C_{20} and C_{22} glycerides, and in the later stages some preferential removal of oleic glycerides. The perinephric and external tissue fats of a number of ewes reared on known diets of varying amount have also been studied by Hilditch & Pedelty (78) who found little variation in the component acids. These lay, respectively, within the following extremes for perinephric and external tissue fats: myristic (1.7 to 3.0, 1.9 to 3.7 per cent), palmitic (23.0 to 26.8, 24.3 to 33.9 per cent), stearic (24.9 to 37.8, 13.5 to 24.6 per cent), hexadecenoic

(0.9 to 2.4, 0.6 to 1.6 per cent), oleic (32.1 to 39.2, 41.2 to 50.8 per cent), octadecadienoic (2.2 to 5.7, 1.5 to 4.9 per cent), and unsaturated C_{20} and C_{22} acids (0.6 to 1.1, 0.5 to 0.9 per cent). Whilst pig and ox depot fats almost invariably contain 30 ± 3 per cent of palmitic acid, the average content of this acid in sheep depot fats is evidently a few per cent less; on the other hand, the general unsaturation of sheep body fats is lower than in those of oxen and pigs, with consequent increase, as compared with the latter, in the proportions of stearic acid.

The depot fats of some Indian oxen were found by Hilditch & Murti (79) to be of an unusually saturated character (iodine values 25 to 31, as compared with the more usual range of 45 to 55). Although their oleic acid content thus fell between the low limits of 26 to 31 per cent of the total fatty acids, the content of stearic acid did not rise above 29 per cent in any instance; but, in the fats of lowest unsaturation, the proportion of palmitic acid rose to over 41 per cent. It would therefore appear that stearic acid may not rise above about 28 to 29 per cent in animal depot fats, any further production of saturated acids being an increase in palmitic acid above the normal limit of about 33 per cent. The cause of the low unsaturation of these Indian ox body fats is uncertain, but may well be connected with the tropical temperatures at which the cattle were reared.

The most detailed statement yet made on the important subject of the fatty acids present in blood lipids resulted from the work of Kelsey & Longenecker (80) on the fatty acids of beef plasma. They found that the total acids were distributed between phospholipids (17.2 per cent), free fatty acids (19.9 per cent), glycerides (16.9 per cent) and cholesterol esters (46.0 per cent). The phospholipid fatty acids were not studied in detail but had characteristics similar to those of the glyceride fatty acids. The component acids of the three groups of acetone-soluble plasma lipids are given (molar per cent) in Table II.

The most striking feature of the data in Table II is perhaps the preponderating amount of octadecadienoic acid in the cholesterol esters. Surprisingly, in view of the fact that linoleic acid has not been detected in more than traces in the diethenoid C_{18} acids of ox depot fats or cow milk fats, this acid was to a large extent the ordinary or "seed-fat" linoleic acid which gives the petrol-insoluble tetrabromo-adduct of m.p. 114° , whilst the ether-insoluble hexabromide of m.p. 185° (characteristic of linolenic acid) was also isolated. The marked unsaturation of the cholesterol esters confirms earlier findings by

other workers. High proportions of octadecadienoic acid are also a feature of the free fatty acids and glyceride fatty acids, but in their saturated acids and in the total proportion of saturated and unsaturated acids the glyceride fatty acids are notably similar to those of ox depot glycerides; the palmitic acid content lies near the usual limits, for depot fats, of 30 ± 3 molar per cent. The results show that, in spite of the great difficulty of isolation of sufficient experimental material (90 litres of blood gave 42 litres of plasma from which 109 gm. of lipids were finally obtained), the detailed study of blood lipids is a topic which merits further attention and will yield much fruitful information.

TABLE II
FATTY-ACID COMPOSITION OF ACETONE-SOLUBLE BEEF-PLASMA LIPIDS
(Molar per cent)

Acids	Free fatty acids	Glyceride fatty acids	Cholesterol ester fatty acids
Myristic acid.....	0.8	0.2
Palmitic acid.....	34.4	33.7	11.1
Stearic acid.....	5.2	22.2	3.3
Arachidic acid.....	2.7	0.5	0.3
Hexadecenoic acid.....	2.6	4.2
Oleic acid.....	40.5	21.3	7.9
Linoleic acid.....	16.4	18.4	61.7
Linolenic acid.....	9.2
Arachidonic acid.....	1.1	2.3

The component acids of milk fat from the Turkish buffalo were found by Heiduschka & Cicekdagi (81) to resemble those of cow milk fat, except for a somewhat lower oleic acid content which was counter-balanced by increased amounts of stearic and arachidic acids. The percentage distribution of the component acids follows: butyric, 4.2; hexoic, 1.3; octoic, 0.4; decoic, trace; lauric, 3.0; myristic, 7.2; palmitic, 25.6; stearic, 16.2; arachidic, 3.2; oleic, 35.2; and linoleic, 2.0. These figures closely resemble those observed by other workers (82) for Indian buffalo milk fat. Buffalo milk constitutes over 8 per cent of the total milk production of Turkey; its fat content is usually higher than that of cow's milk, ranging from 4 to 10 per cent with a mean value of about 7.6 per cent.

COMPONENT ACIDS OF PHOSPHOLIPIDS

The component acids of two specimens of the phospholipids of cow's milk were determined by Hilditch & Maddison (83) by ester fractionation with the following results: myristic (3.2, 5.5 per cent), palmitic (21.0, 13.4 per cent), stearic (7.3, 9.0 per cent), higher saturated acids (as C_{20} and C_{28}) (17.5, 30.9 per cent), hexadecenoic (4.3, 4.9 per cent), oleic (32.5, 23.5 per cent), octadecadienoic (6.4, — per cent), and unsaturated C_{20} and C_{22} acids (7.8, 12.8 per cent). These findings confirm those of Kurtz *et al.* (84) and of Kurtz & Holm (85), namely, that the characteristic lower saturated acids of butter fat are absent from the phospholipid fractions. The composition of the latter is, in fact, not very dissimilar from that of ox liver phospholipids (*v. infra*).

It may be useful here to refer to data obtained by Hilditch & Shorland (86) for the chief component acids of the phospholipid and glyceride fractions of the livers of oxen, pigs, and sheep. In Table III these are compared with typical data for perinephric or external tissue fat glycerides.

The liver glycerides resemble the depot glycerides to some extent, especially in their palmitic acid content, but contain definitely more unsaturated C_{20} and C_{22} acids and hexadecenoic acids, with less stearic acid. The liver phospholipids differ from both types of glyceride in lower proportions of palmitic acid, and of oleic and octadecadienoic acids, whilst the unsaturated C_{20} and C_{22} acids are present in relatively large amounts. Hexadecenoic acid is, in two instances out of three, less abundant in the phospholipids than in the liver glycerides, but, curiously, the stearic acid content of the liver phospholipids exceeds that of the liver glycerides in all cases. The phospholipids of the three animal livers (86) and of cow milk fat (83) exhibit greater resemblances to each other than to any of their corresponding glyceride fractions. It is difficult to find in these data any justification for the assumption, formerly frequently made, that phospholipids play any integral part in the elaboration of glyceride fat in the animal metabolism.

The phospholipids of seeds appear to contain the same acids as the seed glycerides (including any specific to the latter), but in different proportions. A sufficient range of detailed figures is not yet available for any wide generalisation to be made, but apparently the seed phosphatides contain more palmitic (or, sometimes, stearic) acid than the corresponding glycerides, and frequently linoleic acid is the out-

standing component of the phospholipids; hexadecenoic and unsaturated C_{20} and C_{22} acids are usually also present in some quantity. Thus, Diemair *et al.* (88) found the phospholipids of barley, wheat, and oats to contain respectively 14.8, 16.7, and 12.2 per cent of saturated (mainly palmitic) acids and 84.6, 83.3, and 86.1 per cent of unsaturated acids (of respective iodine values 170, 135, and 198)

TABLE III

COMPARISON OF COMPONENT ACIDS OF LIVER PHOSPHATIDES AND GLYCERIDES, AND TYPICAL DEPOT GLYCERIDES, OF OXEN, PIGS, AND SHEEP

	SATURATED ACIDS			UNSATURATED ACIDS		
	C_{14}	C_{16}	C_{18}	C_{18} (-2.0)*	C_{20}	C_{20} & C_{22}
OX						
Liver phosphatide (86)...	1.5	29.7	17.0	1.6	27.7(-2.9)*	21.7(-7.5)*
Liver glyceride (86).....	1.6	32.5	5.0	11.7	40.3(-3.0)	7.4(-6.9)
Depot glyceride† (87)...	2.7	30.4	23.7	1.7	40.6(-2.1)
PIG						
Liver phosphatide (86)...	...	13.4	15.3	5.3	40.3(-2.2)	24.0(-6.5)
Liver glyceride (86).....	0.1	25.8	10.3	9.2	44.1(-2.4)	10.3(-6.8)
Depot glyceride† (77)...	0.9	29.3	17.4	1.8	48.4(-2.3)	1.9(-6.0)
Depot glyceride† (77)...	0.9	26.5	12.8	1.9	54.7(-2.3)	2.9(-6.0)
SHEEP						
Liver phosphatide (86)...	...	13.9	21.7	9.9	28.0(-3.1)	25.8(-7.5)
Liver glyceride (86).....	0.3	24.2	12.5	5.3	44.1(-2.8)	13.7(-7.5)
Depot glyceride† (78)...	2.3	26.2	27.1	1.0	42.0(-2.2)	1.1(-6.0)
Depot glyceride† (78)...	3.1	28.3	13.5	0.6	52.7(-2.1)	0.7(-6.0)

* Mean unsaturation indicated by atoms of hydrogen per molecule short of saturation (e.g., -2.1, etc.).

† Perinephric fat.

‡ External tissue fat.

which readily yielded the crystalline bromo adduct (m.p. 114°) of seed-fat linoleic acid. Hilditch & Pedelty (89) found the percentage distribution of the component acids of soybean phospholipids to be as follows: palmitic, 13; stearic, 4; hexadecenoic, 10; oleic, 10; linoleic, 53; linolenic, 4; and unsaturated C_{20} acid, 5, whilst that of rape seed phospholipids was: myristic, 1; palmitic, 9; behenic, 1; hexadecenoic, 2; oleic, 23; linoleic, 44; and erucic, 20. Diemair & Weiss (90) observed that the phospholipids of lupin seeds contained 15 per cent of saturated acids (palmitic and traces of arachidic), the remainder being oleic, linoleic, and linolenic acids.

COMPONENT GLYCERIDES OF NATURAL FATS

The determination of the proportions of the various mixed glycerides present in natural fats has continued to receive attention. A summary of the chief results in this field down to 1935 was given in Vol. V of these *Reviews* (91) and recent progress was reported in Vol. IX (7). The most fruitful recent development has been the preliminary separation of a fat into several portions of varying solubility in acetone; each fraction is naturally a considerably simpler mixture of mixed glycerides than the whole fat, and usually not more than two of the main general groups (fully saturated, monounsaturated-disaturated, diunsaturated-monosaturated and completely unsaturated glycerides) predominate in any one portion. Determination of the component acids, fully saturated glycerides, etc., present in each fraction therefore frequently leads to its glyceride composition being ascertained with some degree of precision, and therefrom that of the original fat. This procedure has now been applied to a fairly large number of vegetable fats in addition to those previously reported, whilst typical ox, pig, and sheep depot fats and cow butter fat have been examined by the same method. A recent extension of the process, in which fats liquid at ordinary temperatures have been separated into a number of fractions by crystallisation at temperatures down to -40° from acetone cooled by addition of solid carbon dioxide, has permitted data to be obtained by similar means in the cases of cottonseed, olive, and some other oils.

VEGETABLE FATS

The following solid fats have been investigated by Hilditch and co-workers in addition to those already reported (7): neem oil (47), virola and kombo fats (28), *Allanblackia* fats (92), palm oils (93), Baku (*Mimusops Heckelii*) fat (94), and *Garcinia* fats (95). In all these fats the usual strong tendency to "even distribution" of the fatty acids throughout the glyceride molecules was evident, although in one or two instances (e.g., the palm oils) this generalisation was slightly less marked than usual in some respects. The seed fat of *Garcinia indica* [component acids: myristic (1.2 per cent), palmitic (5.3 per cent), stearic (52.0 per cent), oleic (41.5 per cent)] had been similarly investigated by Vidyarthi & Dasa Rao (96). Vidyarthi & Mallya (97) found that the glycerides of sapota fat [component acids: lauric (1.6 per cent), myristic (6.2 per cent), palmitic (12.6 per cent), stearic (12.0 per cent), oleic (66.2 per cent), and linoleic (1.4 per

cent)] also conformed with the rule of even distribution. It is of interest to give a summary (Table IV) of the chief component mixed

TABLE IV
CHIEF COMPONENT GLYCERIDES OF SOME VEGETABLE FATS

Vegetable fat	Percentage distribution of chief glycerides
Palm oil* (93).....	Oleodipalmitin, 43; palmitodiolein, 31; oleopalmitostearin, 11
Palm oil† (93).....	Oleodipalmitin, 31; palmitodiolein, 41; oleopalmitostearin, 10
Neem oil (47).....	Stearodiolein, 34; palmitodiolein, 26; oleolinoleins, 22
Virola fat (28).....	Trimyristin, 43; laurodimyristin, 31
Kombo fat (28).....	Tetradecenodimyristin, 33; trimyristin, 24; laurodimyristin, 17
<i>Allanblackia floribunda</i> (92) ..	Oleodistearin, 76; stearodiolein, 15 to 16
<i>Allanblackia parviflora</i> (92) ...	Oleodistearin, 60; stearodiolein, 27 to 30
Baku fat (94)	Stearodiolein, 41 to 47; oleodistearin, 26 to 32; palmitodiolein, 6 to 14
<i>Garcinia morella</i> (95).....	Stearodiolein, 47; oleodistearin, 46
<i>Garcinia indica</i> (95).....	Oleodistearin, 68; stearodiolein, 20
<i>Garcinia indica</i> (96).....	Oleodistearin, 59; stearodiolein, 21; oleopalmitostearin, 14
Sapota fat (97).....	Palmitodiolein, 36; stearodiolein, 28; myristodiolein, 23

* Cameroons plantation palm oil. Component acids: myristic (1.1 per cent), palmitic (45.1 per cent), stearic (4.1 per cent), hexadecenoic (0.8 per cent), oleic (38.6 per cent), linoleic (10.3 per cent).

† Grand Bassa native palm oil. Component acids: myristic (0.6 per cent), palmitic (37.6 per cent), stearic (3.7 per cent), hexadecenoic (1.4 per cent), oleic (50.3 per cent), linoleic (6.4 per cent).

glycerides reported in each of these solid or semi-solid vegetable fats which have been studied by the acetone-crystallisation procedure.

The use of crystallisation from acetone to effect considerable preliminary resolution of mixed glycerides has been extended to liquid fatty oils in several ways. In the case of niger seed oil [*Guizotia abyssinica*, component acids: myristic (2 per cent), palmitic (5 per cent), stearic (2 per cent), oleic (39 per cent), and linoleic (52 per cent)] Vidyarthi & Mallya (98) first separated the small amount of glycerides insoluble in acetone at 0°, and then added bromine to the remaining glycerides and subsequently separated the bromo adducts

into several fractions by crystallisation from various solvents; they found the chief components of the acid to be oleodilinolein (40 per cent), linoleodiolein (30 per cent), and palmito-oleolinolein (11 per cent). Gunde & Hilditch (99) studied groundnut, almond, and olive oils by first isomerising ("elaidinising") each oil with selenium at 220°, and then crystallising the semi-solid elaidinised products from acetone at 0° or room temperature. The isomerisation procedure involves some loss of linoleo-glycerides by polymerisation, but it was possible to show that "even distribution" dominated the glyceride structure of each oil, the chief components of which were: groundnut,—palmito-(and other monosaturated)-oleolinoleins (45 per cent), palmito-(and other monosaturated)-dioleins (11 per cent), linoleodioleins (24 per cent), and triolein (19 per cent); almond,—palmito-(and myristo-) dioleins (17 per cent), linoleodioleins (52 per cent), triolein (31 per cent); olive (Turkish),—palmito-, etc., -dioleins (38 per cent), linoleodioleins (26 per cent), triolein (31 per cent); olive (Tuscany, 1939),—palmito-, etc., -dioleins (54 per cent), oleo-palmito- (etc.) -linoleins (6 per cent), and linoleodioleins (38 per cent).

Finally, as already mentioned, the acetone crystallisation procedure has been used for the direct separation of some of the constituents of liquid fats by employing temperatures down to -40°. By this means, Hilditch & Maddison (100) studied the same two olive oils as those used by Gunde & Hilditch (*v. supra*), and obtained data of a similar order: chief components of the olive oil (Turkish,—palmito- (and other monosaturated)-dioleins (45 per cent), linoleodioleins (26 per cent), triolein (29 per cent), and of the olive oil (Tuscany, 1939),—palmito-(and other monosaturated)-dioleins (57 per cent), linoleodioleins (34 per cent), oleopalmito-(etc.)-linoleins (4 per cent), and triolein (5 per cent). It should be noted that these oils represent two types of olive oil differing somewhat widely in fatty acid composition, the respective percentages for the Turkish and Tuscany oils being myristic, 0.5, 1.2; palmitic, 10.0, 15.6; stearic, 3.3, 2.0; hexadecenoic, 1.0, 1.6; oleic, 76.5, 64.6; and linoleic, 8.6, 15.0. Riemen-schneider *et al.* (101) studied the separation of cottonseed oil glycerides both by distillation of the oil in a molecular still and by low-temperature crystallisation, and stated that appreciable separation of the glycerides was effected by the latter, but not by the former method. Hilditch & Maddison (102) separated cottonseed oil into six fractions by crystallisation from acetone between 0° and -35°, and from the

component acids in each fraction deduced the presence in the original oil of palmito-oleolinoleins (35 to 40 per cent), palmitodilinoleins (over 20 per cent), nearly 30 per cent of triunsaturated glycerides (mainly oleodilinoleins), and about 13 per cent of oleo- or linoleo-dipalmitins.

ANIMAL FATS

The crystallisation procedure in the determination of constituent glycerides has been applied in the Liverpool laboratories to a comprehensive range of ox, pig, and sheep depot fats and also to a cow milk fat (103). The depot fats examined included the perinephric fat of an English ox (87), two Indian cow depot fats (79), the perinephric and outer back fats from a pig (104), and the perinephric and external tissue fats of a sheep (105). The new procedure enabled the limiting values for the chief mixed glycerides present to be considerably narrowed as compared with earlier studies; the limit of uncertainty for any particular component (e.g., steardiolein or triolein) was reduced to not more than 5 to 10 units per cent, and this uncertainty was solely due to lack of evidence as to how far the tri- C_{18} glycerides in the most soluble portions of the fats consisted of steardiolein or triolein. Since other evidence points to the presence of triolein in appreciable proportions in any of the fats in question being extremely unlikely, it was possible without likelihood of any serious error to presume the triolein contents to be the minimum compatible with the data, and to give approximate figures for the component glycerides present.

In the depot fats, the data thus obtained give strong support to the hypothesis put forward earlier by Banks & Hilditch (106), namely, that in the relatively stearic-rich depot fats of land animals the stearglycerides have been produced by hydrogenation of preformed oleoglycerides (e.g., palmitodiolein or triolein). This is illustrated by the data for the component acids and glycerides of the seven depot fats in Table V. In this table the fats are arranged in roughly decreasing order of general unsaturation; this, it will be observed, is to a large extent accompanied by increasing stearic acid content, although the limit of the latter appears to be about 28 per cent, beyond which further diminution in oleic acid is apparently compensated for by increases beyond the normal proportion of palmitic acid (cf. p. 87).

Experimental conditions in this type of investigation render it possible to deal only with three component acids at a time, i.e., the major components palmitic, stearic, and "oleic" (the terms "oleic,"

TABLE V

COMPONENT ACIDS AND GLYCERIDES OF PIG, SHEEP, AND OX DEPOT FATS
(Molar per cent)

Components	Ewe (Ex- ternal fat) (105)	Pig (Back fat) (104)	Pig (Peri- neph- ric fat) (104)	Ewe (Peri- neph- ric fat) (105)	Ox (Eng- lish) (87)	Cow (Cali- cut) (79)	Cow (Bom- bay) (79)
<i>Component acids</i>							
Myristic (+C ₁₂)	3.8	1.3	1.8	3.0	2.6	5.7	5.9
Palmitic	31.5	29.0	31.1	27.1	33.4	33.4	40.8
Stearic	14.5	13.8	17.6	25.6	21.4	27.9	25.5
Arachidic	0.4	1.5	1.3	0.5	0.7
Hexa-(+tetra-)decenoic ..	1.2	2.7	2.4	2.0	2.5	1.9	2.8
Oleic	45.8	43.9	40.6	37.1	35.2	29.0	22.9
Octadecadienoic	2.1	7.2	5.3	3.3	3.5	1.5	1.1
Unsaturated C ₂₀₋₂₂	0.7	2.1	1.2	0.4	0.1	0.1	0.3
<i>Component glycerides (ap- prox.)</i>							
Fully saturated:							
Tripalmitin	Trace	1	Trace	3	3
Dipalmitostearin	3	2	4	4	8	16	23
Palmitodistearin	2	2	5	10	6	12	10
Tristearin	Trace
Mono-"oleo"-disaturated:							
"Oleo"-dipalmitin	13	5	9	5	15	11	18
"Oleo"-palmitostearin	28	27	34	41	32	38	34
"Oleo"-distearin	1	2	2	2
Di-"oleo"-monosaturated:							
Palmitodi-"olein"	46	53	40	25	23	17	11
Stearodi-"olein"	7	7	5	13	11	3	1
Tri-"oleins"	0	3	3	0	0	0	0

"oleo"—or "olein" imply that both unsaturated C₁₈ acids, and not merely oleic itself, are included). In the component glycerides, therefore, the minor amounts of myristic and lauric acids are included with palmitic (as are also those of tetra- and hexa-decenoic acids which yield myristo- or palmito-glycerides on hydrogenation); traces of arachidic are included with stearic, and of unsaturated C₂₀ and C₂₂ acids with "oleic." The palmitic acid content of the Bombay cow fat, and also the combined palmitic and myristic acid content of the Calicut cow fat, is abnormally high, and this to some extent compli-

cates comparison as regards the development of mixed stearo-glycerides with increasing general saturation in the fats. Nevertheless, it is instructive to compare the relative proportions of palmitodi-"oleins," "oleo"-palmitostearins and palmitodistearins as the proportion of stearic acid in the whole fat increases (Table VI).

TABLE VI
RELATIVE PROPORTIONS OF PALMITODI-"OLEINS," "OLEO"-PALMITOSTEARINS,
AND PALMITODISTEARINS

Components	Pig (Back fat)	Ewe (Ex- ternal fat)	Pig (Peri- neph- ric fat)	Ox (Eng- lish)	Ewe (Peri- neph- ric fat)	Cow (Call- cut)	Cow (Bom- bay)
C₁₈ fatty acids:							
Stearic	13.8	14.5	17.6	21.4	25.6	27.9	25.5
Oleic+octadecadienoic	51.1	47.9	45.9	38.7	40.4	30.5	24.0
Palmitodi-C₁₈ glycerides (incre- ments per cent):							
Palmitodi-"oleins"	53	46	40	23	25	17	11
"Oleo"-palmitostearins	27	28	34	32	41	38	34
Palmitodistearins	2	2	5	6	10	12	10
	82	76	79	61	76	67	55
Palmitodi-C₁₈ glycerides (per cent):							
Palmitodi-"oleins"	65	61	51	38	33	25	20
"Oleo"-palmitostearins	33	37	43	52	54	57	62
Palmitodistearins	2	2	6	10	13	18	18

The progressive change in the relative proportions of the three types of palmitodi-C₁₈ glycerides, shown at the foot of Table VI, is an extremely strong argument in favour of the hydrogenation hypothesis already mentioned. A somewhat similar comparison may be made in regard to the relative proportions of "oleo"-dipalmitin and dipalmitostearin in the dipalmitomono-C₁₈ group of glycerides. Here, however, the total proportion of the dipalmito-glycerides is partly dependent upon the palmitic acid content in the whole fats; the latter rises considerably above the average in the Indian cow depot fats with consequent increase in the amount of dipalmito-glycerides at the expense of the monopalmitodi-C₁₈ group. Apart from increased proportions of dipalmito-glycerides from this cause, however, a tendency has previously been observed (104) for this group to increase in amount with

increasing general saturation in the fats as a whole. The relevant data on this aspect for the seven fats for which information is now available are summarised in Table VII. In this case, since the minor quantities

TABLE VII
RELATIVE PROPORTIONS OF "OLEO"-DIPALMITIN AND DIPALMITOSTEARIN

Components	Pig (Back fat)	Ewe (Ex- ternal fat)	Pig (Peri- neph- ric fat)	Ox (Eng- lish)	Ewe (Peri- neph- ric fat)	Cow (Call- cut)	Cow (Bom- bay)
Fatty acids:							
Palmitic (+C ₁₄ , C ₁₂)	30.3	35.3	32.9	36.0	30.1	39.1	46.7
Stearic	13.8	14.5	17.6	21.4	25.6	27.9	25.5
Total unsaturated	55.9	49.8	49.5	41.3	42.8	32.5	27.1
Dipalmitomono-C₁₈ glycerides (increments per cent):							
"Oleo"-dipalmitins	5	13	9	15	5	11	18
Dipalmitostearins	2	3	4	8	4	16	23
	7	16	13	23	9	27	41
Dipalmitomono-C₁₈ glycerides (per cent):							
"Oleo"-dipalmitins	71	81	69	65	56	41	44
Dipalmitostearins	29	19	31	35	44	59	56

of lower saturated acids present are perforce included with palmitic acid in the evaluation of the main component glycerides, comparison is made with the combined amount of palmitic, myristic, and lauric acids in the total acids of the fats.

The total amount of dipalmito-glycerides in the depot fats bears a general, though somewhat irregular, relationship to the proportion of palmitic acid present in the whole fat; but the relative proportions of "oleo"- and stearo-dipalmitins are governed entirely, as in the mono-palmitodi-C₁₈ glycerides, by the proportion of stearic and unsaturated acids present. Thus the view is again supported that production of the more saturated component glycerides involves hydrogenation of preformed "oleo"-glycerides,—in this case "oleo"-dipalmitins.

The acetone crystallisation procedure has also been applied (103) to the complex case of a cow milk fat. Here, although the results were naturally less definite than in fats with simpler mixtures of component acids, it was possible to show that the characteristic lower saturated

acids of butter were present only once, or at most twice, in any triglyceride molecule. Twenty-two to 30 per cent of the milk fat consisted of glycerides with one radical each of oleic, palmitic, and one of these lower acids; nearly 40 per cent was made up of four types of mixed glyceride: oleo-mono-C₄₋₁₄-palmitins, oleopalmitostearins, palmitodioleins and oleo-mono-C₄₋₁₄-stearins. About 20 per cent was fully saturated glycerides (chiefly mono-C₄₋₁₄-palmitostearin and di-C₄₋₁₄-monopalmitin). Palmitic acid was present in at least 70 per cent and the lower saturated acids in about 55 to 60 per cent of the butter glyceride molecules.

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THE CHEMISTRY OF THE STEROIDS

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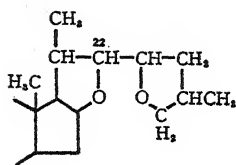
In view of the comprehensive article on the sterols by Heilbron & Jones (1), of the treatment by Kendall (2) and by Kamm & Pfiffner elsewhere in this Volume of the various groups of steroid hormones, and also through limitation of space, this review is restricted to the steroid sapogenins, the cardiac glycosides and aglycones, and the toad poisons. These sections of the subject have not been treated in this series of reviews since they were briefly considered by Schoenheimer & Evans (3) and Kellaway (4). Apart from an early report by Cook (5) and the appropriate chapters, already partially obsolete, of the monographs by Lettré & Inhoffen (6), Fieser (7), and Sobotka (8), there is available¹ no survey of these substances which have been extensively investigated, mainly in American laboratories, during the last three years.

Before passing on to the sections selected for review the total synthesis of *d*-equilenin and its three stereoisomerides by Bachmann, Cole & Wilds (9) must be mentioned as perhaps the most spectacular achievement yet accomplished in steroid chemistry. Of general interest is the important paper by Dimroth & Jonsson (10) indicating that rings C and D of the natural steroids possess the *trans*-configuration, whilst the summary of ultraviolet absorption spectra of steroids compiled by Dannenberg (11) should be of general utility. Attention may also be directed to the work of Butenandt, Mamoli, and co-workers on the biochemical oxidation and reduction of steroids (12).

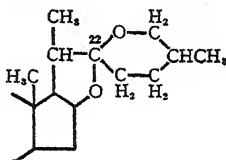
THE STEROID SAPOGENINS

The sapogenins (I) are now known to be C₂₇-compounds (13, 14, 15), the first indication of their steroid structure being the isolation

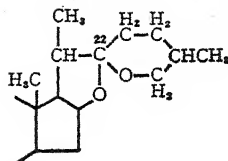
¹ Since the manuscript of this review was completed, Gilman's *Organic Chemistry* has become available and the reviewer has been able to see (through the kindness of Professor Dr. L. Ruzicka) a copy of the *Annual Report of the Chemical Society* for 1940: this contains an article on steroids by F. S. Spring, which includes a short section on sapogenins (37, 332-63, 1940).



IV



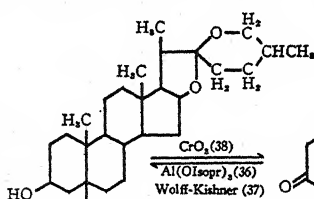
V. Normal



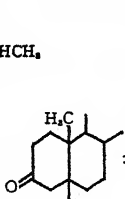
VI. Iso

according to: (a) the number, position, and configuration of nuclear hydroxyl groups; (b) the configuration, *cis* or *trans*, of rings A and B; and (c) the configuration, normal or iso, at C-22. The various sapogenins are set out in Table I.

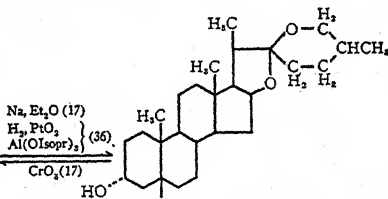
Sapogenins whose isomerism is confined to epimerism at C-3, must yield the same 3-keto compound; thus sarsasapogenin (VII) and episarsasapogenin (VIII) both afford sarsasapogenone (IX), reduction of which with aluminum isopropylate regenerates equal quantities of the epimeric sapogenins. The direct epimerisation of sarsasapogenin with sodium in amyl alcohol has also been accomplished (36).



VII. Sarsasapogenin

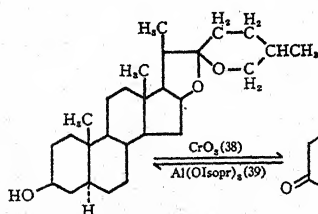


IX

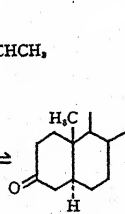


VIII. Episarsasapogenin

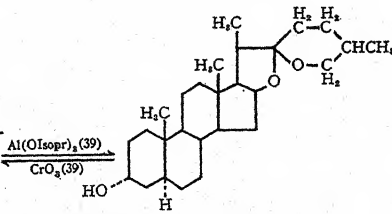
Similarly, tigogenin (X) and epitigogenin (XI) both afford tigo-genone (XII), which is reduced by the Meerwein-Ponndorf reagent to a mixture of the two epimerides.



X. Tigogenin



XII



XI. Epitigogenin

Not only epimers at C-3, but also other sapogenins belonging to the same stereochemical series (e.g., tigogenin, gitogenin, chlorogenin)

TABLE I
SAPOGENINS

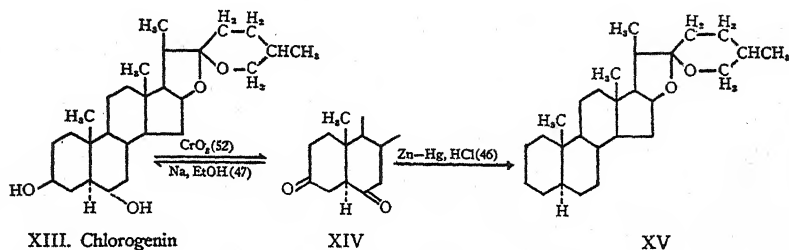
Name	Formula	Melting point °C.	Optical rotation $[\alpha]_D$	Configuration A/B	Position and configuration of OH-groups	Configuration at C ₂₂	Source*
Sarsapogenin (Parigenin)	C ₂₇ H ₄₄ O ₃	199	-75°	cis	3(β)	normal	<i>Radix sarsaparilla</i> (Vera Cruz) (17); <i>Asparagus officinalis</i> (40)
Smilagenin (Isosarsapogenin)	C ₂₇ H ₄₄ O ₃	184	-69°	cis	3(β)	iso	<i>Radix sarsaparilla</i> (Jamaica) (17, 34)
Episarsapogenin	C ₂₇ H ₄₄ O ₃	207	-71°	cis	3(α)	normal	Synthetic (17, 36, 41)
Neotigogenin (Isotigogenin)	C ₂₇ H ₄₄ O ₃	201	-65°	trans	3(β)	normal	<i>Chlorogalum pomeridianum</i> (42, 43)
Tigogenin	C ₂₇ H ₄₄ O ₃	208	-49°	trans	3(β)	iso	<i>Digitalis purpurea</i> , <i>Digitalis lanata</i> , <i>Chlorogalum pomeridianum</i> (44, 45, 46)
Epitigogenin	C ₂₇ H ₄₄ O ₃	245		trans	3(α)	iso	Synthetic (39)
Nitogenin	C ₂₇ H ₄₄ O ₃	201	-112°	?	?	?	<i>Balanites aegyptica</i> (49)
Chlorogenin†	C ₂₇ H ₄₄ O ₄	276	-46°	trans	3(β), 6(α)	iso	<i>Chlorogalum pomeridianum</i> (44, 46)
β-Chlorogenin	C ₂₇ H ₄₄ O ₄	248		trans	3(β), 6(β)	iso	Synthetic (47)
Neochlorogenin†	C ₂₇ H ₄₄ O ₄			cis	3,6	iso	<i>Digitalis purpurea</i>
Gitogenin	C ₂₇ H ₄₄ O ₄	272	-61°	trans	2,3	iso	<i>Digitalis purpurea</i> , <i>Digitalis germanicum</i> , <i>Digitalis purpurea</i>
Liligenin	C ₂₇ H ₄₄ O ₄	246		?	2,3 or 3,4	?	<i>Chlorogalum pomeridianum</i> (46, 48)
Digitogenin	C ₂₇ H ₄₄ O ₆	283		?trans	2,3,6	iso	<i>Lithium rubrum magnificum</i> (40)
Diosgenin (Δ ⁸)	C ₂₇ H ₄₂ O ₅	208	-129°		3(β)	iso	<i>Digitalis purpurea</i> <i>Dioscorea tokoro</i> , <i>Dioscorea villosa</i> , <i>Aleuris fatinosa</i> , <i>Trillium erectum</i> (40, 49, 50, 51)

* For references prior to 1935, see Fieser (7).

† The only outstanding objection to formulation of chlorogenin as a 3,6-diol is the isolation (52) by oxidative fission of an acid said to be different from digitogenic acid; it should, of course, be identical, but see 111b.

‡ Known only as the 3,6-diketone (47, 54).

should furnish the same desoxy-compound. Thus chlorogenin (XIII) by oxidation to chlorogenone (XIV) and subsequent reduction by Clemmensen's method gives the same desoxy-compound (XV)² as does tigogenin (54) by similar treatment.

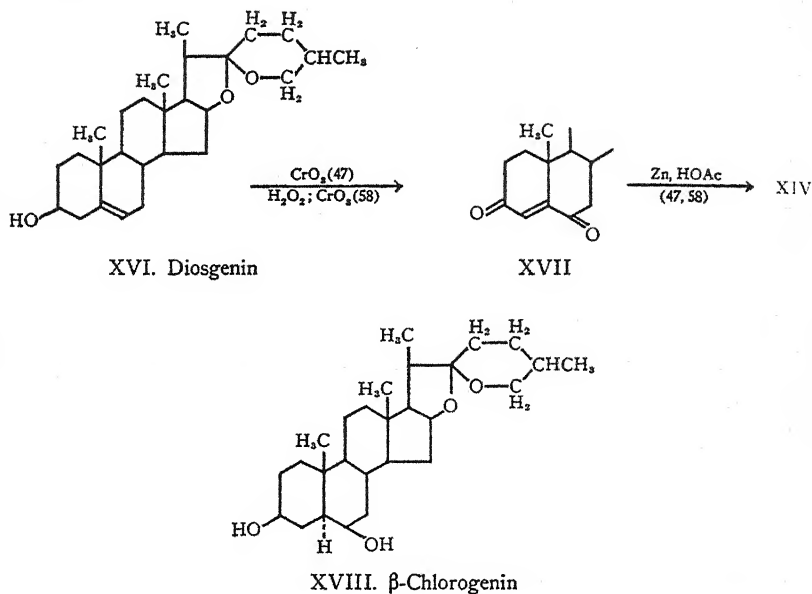


By taking advantage of the observation (55) that Wolff-Kishner reduction of steroid 3-semicarbazones yields mainly the carbinols epimeric at C-3, whereas the 6-, 7-, and 12-semicarbazones furnish methylene groups, the disemicarbazone of chlorogenone (XIV) has been converted directly to tigogenin (56), which confirms the above relationship.

Chlorogenin was provisionally regarded as a 3(α),12-dihydroxygenin by Noller (52). Marker & Rohrmann reported (46) the formation of an insoluble digitonide, and nonepimerisation with hot sodium amylate (47), and because chlorogenone (XIV) gave a "pyridazine"³ suggested the 3(β),6-dihydroxy formula XIII. This has been confirmed by the conversion of diosgenin (XVI) to chlorogenone (XIV) via the Δ^4 -diketone (XVII) by oxidation with hydrogen peroxide (58), and (47) by the general method of Windaus (59, 60) for which an improved procedure was given (61).

² Jacobs & Fleck (38) obtained a compound, m.p. 267°, described as desoxytigogenin, by Clemmensen reduction of tigogenone (XII) in very poor yield. The melting point of compound XV when described as "desoxychlorogenin" is given by Marker & Rohrmann (46) as 177-178° and (54) as 172°; when called "desoxytigogenin" the melting point is given by Marker *et al.* (28, 54) as 173-174°. It may be pointed out that identities described by Marker and his co-workers always depend solely on mixed melting point determinations (which are notoriously unreliable amongst steroids) unsupported by measurement and comparison of optical rotations.

³ The use of pyridazines to characterise steroid 3,6-diketones has been criticised because their molecular weights are some ten times too large: in the case of the pyridazine from cholestane-3,6-dione it has however been shown that the compound is essentially monomolecular in various solvents (57).



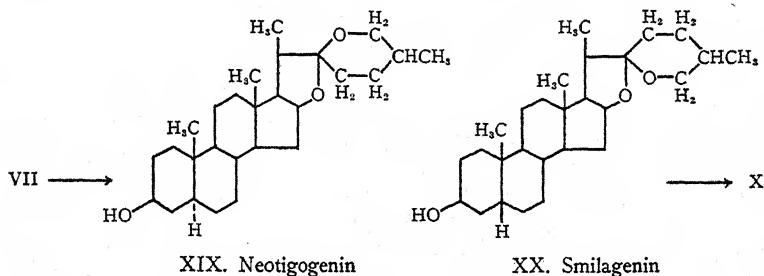
A comparison of the reduction products of chlorogenone and cholestane-3,6-dione indicates that the configurations of the two hydroxyl groups in chlorogenin (XIII) are 3(β) and 6(α). Reduction of these compounds with sodium and alcohol gives respectively natural chlorogenin and cholestane-3(β),6(α)-diol, m.p. 216° (47, 62). Catalytic (platinum oxide catalyst) reduction of chlorogenone, cholestane-3-ol-6-one (63), and cholestane-6-ol-3-one (64) gives respectively the new synthetic sapogenin, β -chlorogenin (XVIII), and cholestane-3(β),6(β)-diol, m.p. 190° (47). The last-named is oxidised (as the diacetate) to allohyodesoxycholic acid in which both hydroxyl groups are known to possess the β -configuration.

The nuclear structure (58, 101) of diosgenin (XVI), the only unsaturated sapogenin, is analogous to that of cholesterol. Numerous reactions of cholesterol have been applied successfully to diosgenin (28, 70). 3-Chlorodiosgenin, from diosgenin (XVI) and phosphorus pentachloride, is reduced to a 3-chlorotigogenin which is not identical with the 3-chlorotigogenin from tigogenin (28): these chloro-compounds are probably analogous to the α - and β -cholesteryl chlorides.

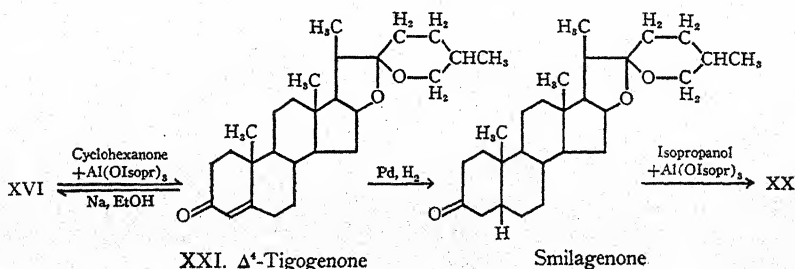
Contradictory results have been reported for the behaviour of chlorogenin (46, 52), gitogenin and digitogenin (29, 66), and diosgenin (50, 65) with digitonin. Noller (67) has determined the values

of the solubility products of various steroid digitonides, and has emphasised the need for caution in using digitonide precipitability as diagnostic of 3(β)-hydroxyl-groups in sapogenins; the solubility products vary so widely that qualitative tests for precipitation are without meaning unless both epimerides can be compared.

The assignments of ring-configuration A/B made in Table I are supported by interconversions of sapogenins of the *cis*- and *trans*-series. Thus sarsasapogenin (VII) by oxidation to sarsasapogenone (IX), introduction of Δ^4 -unsaturation by bromination followed by elimination of hydrogen bromide with pyridine, and reduction of the resulting Δ^4 -3-ketone gives neotigogenin (XIX) (68). Smilagenin (XX) by a precisely similar sequence of reactions furnishes tigogenin (X) (69).

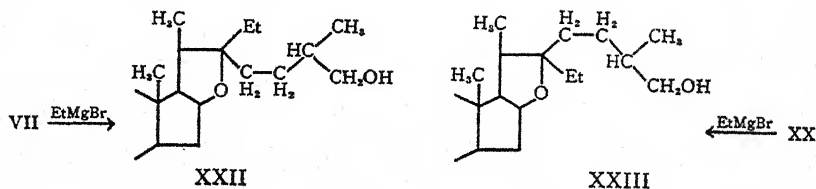


Reactions used to interconvert sapogenins must not cause alteration in the side-chain with consequent possible inversion at C-22; this condition is clearly complied with in the transformation of Δ^4 -tigogenone (XXI) to smilagenin (XX) (70). Since Δ^4 -tigogenone (XXI), [obtained from diosgenin (XVI) and reconvertible to this (70)], can be reduced to tigogenin (70), this conversion is in effect the reverse of the transformation $XX \rightarrow X$.

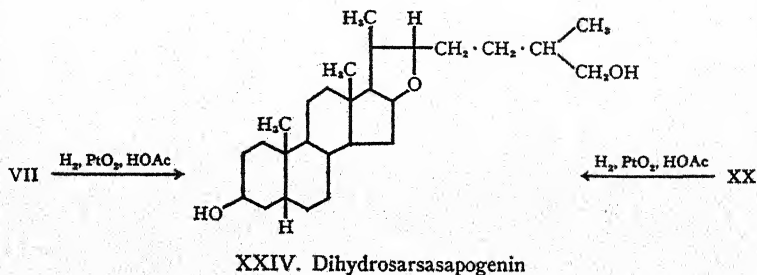


The side-chain structure present in the sapogenins shows considerable stability to alkaline reagents (13, 28, 56, 71), but is reactive in acid media (71). Certain sapogenins (smilagenin, tigogenin, gitogenin, chlorogenin, digitogenin, and diosgenin) are unaltered by prolonged treatment with hot alcoholic hydrochloric acid (64, 66). By contrast, brief treatment under the same conditions converts sarsasapogenin (VII) to smilagenin (XX) (34), and neotigogenin (XIX) to tigogenin (X) (69). Taken together with the transformations VII \rightarrow XIX and XX \rightarrow X given above, it follows that sarsasapogenin and neotigogenin have the normal side-chain configuration (V), whilst smilagenin, tigogenin, gitogenin, chlorogenin, digitogenin, and diosgenin all possess the opposite iso-configuration (VI).

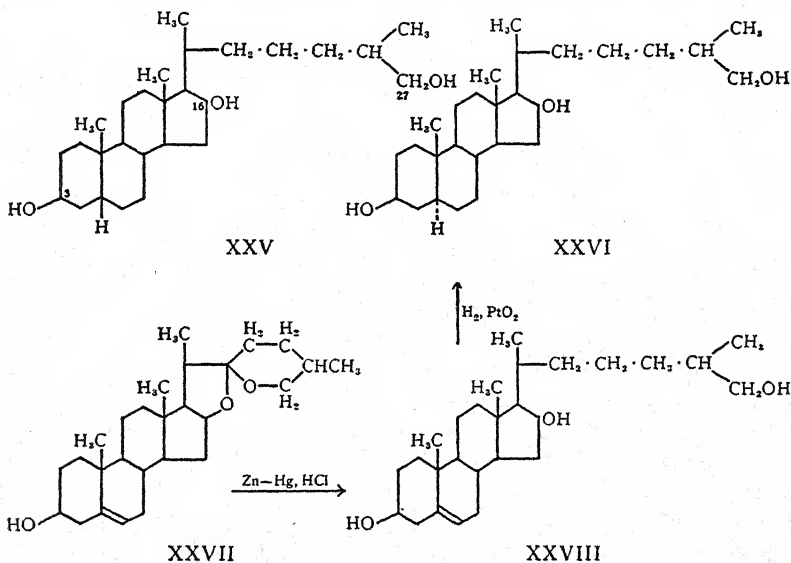
Each pair of sapogenins, isomeric at C-22, should yield isomeric and not identical substances by fission of the six-membered oxide ring. With ethyl magnesium bromide, sarsasapogenin (VII) and smilagenin (XX) yield 22-ethyl-27-ols (XXII, XXIII), which are very similar but not identical (72). The isomer represented by formula XXII on mild oxidation with chromium trioxide gives a C₂₉-3-keto-monocarboxylic acid (72).



The sapogenins are readily reduced to dihydro-compounds by Adams catalyst in acidic but not in neutral media (34, 36, 47, 54, 73, 74, 75). Sarsasapogenin (VII) and smilagenin (XX) give the same dihydrosapogenin (XXIV) (34) and not isomerides.



The dihydrosapogenins furnish 3,27-diacyl derivatives which regenerate the parent compounds by hydrolysis; 3-monoacyl compounds are obtained by hydrogenation of the 3-acylsapogenins. Reduction of the sapogenins with the Clemmensen reagent gives erratic results (34, 36, 54, 73, 74, 75, 76). When crystalline products are obtained, these are tetrahydrosapogenins, formulated in the cases of sarsasapogenin and smilagenin, which give the same substance (34), as coprostane-3(β),16,27-triol (XXV).⁴ This compound furnishes only diacyl derivatives under the usual conditions, but with acetic anhydride at 200° gives a triacetate, the 16-acetoxy group of which resists hydrolysis by hot 5 per cent alcoholic potassium hydroxide (77). It is noteworthy that cholestane-3(β),16,27-triol (i.e., tetrahydrotigogenin) (XXVI), which could not be prepared by direct Clemmensen reduction of tigogenin (54) but is obtained from diosgenin (XXVII) by reduction to tetrahydrodiosgenin (XXVIII) and hydrogenation of this



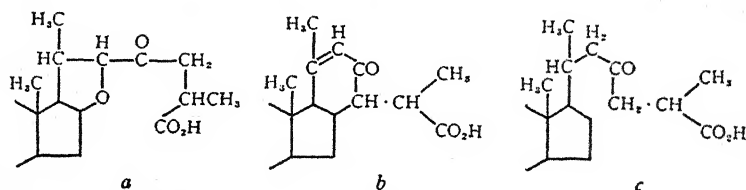
(28), readily forms triacyl derivatives; this is also true of XXVIII, and the ready hydrolysis of 16-acyloxy compounds has been invoked

⁴ Indirect evidence that fission of the six-membered oxide ring occurs in the formation of the dihydrosapogenins (XXIV) is provided by the fact that XXIV is not reduced by the Clemmensen reagent to XXV, but recovered unaltered; i.e., the spiro-structure of the sapogenin, which appears in some respects to be equivalent to an activating carbonyl group, is no longer present.

to account for the oxidation products of derivatives of sarsasapogenin (78) and tigogenin (79) (see p. 117).

Oxidation of the sapogenins and dihydrosapogenins with chromium trioxide under varying conditions gives rise to numerous products. The products derived from sarsasapogenin are shown in Table II. Similar products have been obtained with tigogenin (29, 54, 80).

Tschesche & Hagedorn (14) originally gave tigogenoic acid (as XXIX) the γ -ketoacid structure (a), and Fieser & Jacobsen (27, 30) adopted a similar structure for sarsasapogenoic acid (XXIX); both acids appear to react with hydroxylamine only under rather drastic conditions to give dioximes (27, 30, 80). Anhydrosarsasapogenoic acid (XXX or XXXI) is an α,β -unsaturated ketone (30, 81). Its structure as (b) was regarded by Fieser & Jacobsen as secure, whilst anhydrotetrahydrosarsasapogenoic acid (XXXII) was given the γ -ketoacid structure (c) (30). Later, the optical properties of the acid showed (c) to be inadmissible, and Fieser (32) suggested the con-



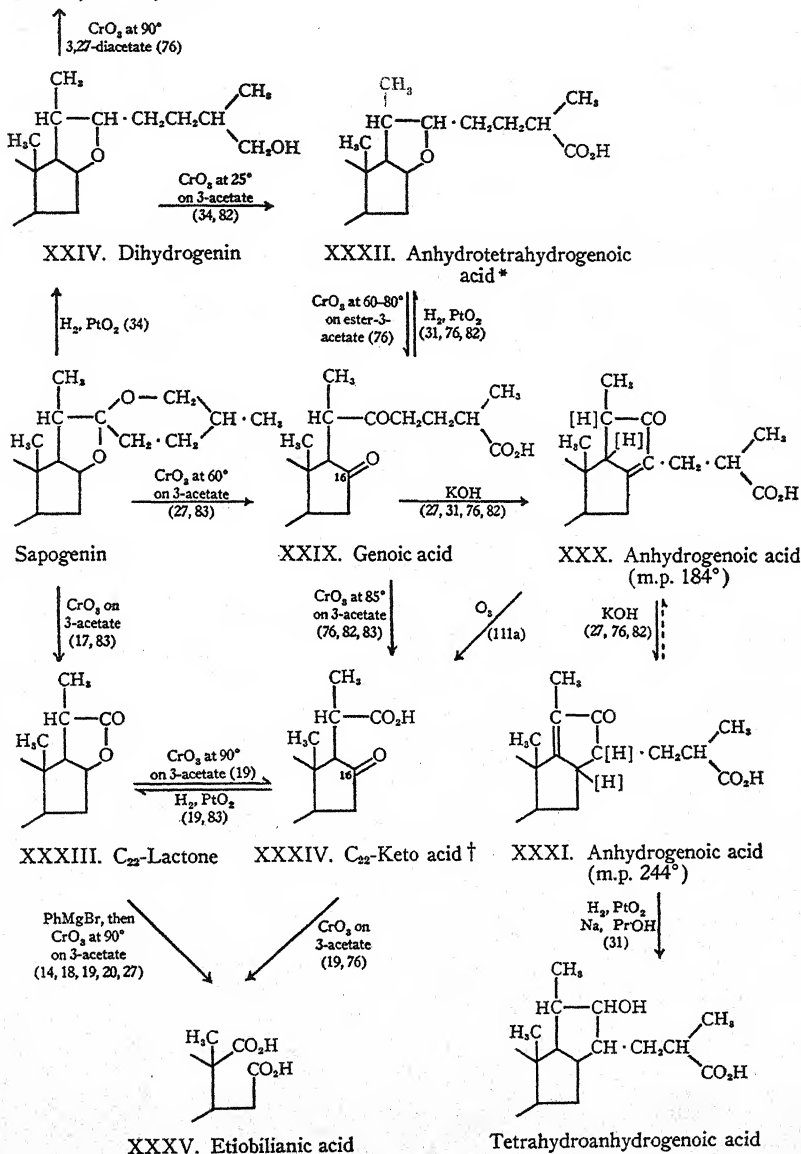
stitution XXXII, subsequently proved to be correct by Marker (82) who prepared the acid from dihydrosarsasapogenin-3-monoacetate (XXIV) by oxidation with chromium trioxide at 25°. The 3-monoacetate of dihydropigogenin similarly gave anhydrotetrahydropigogenoic acid (80), whilst dihydrochlorogenin gave 3,6-dehydroanhydrotetrahydrochlorogenoic acid (75). The identity (XXIV \rightarrow XXXII and XXIX \rightarrow XXXII) disposes of Fieser's principal ground of criticism (32) of Marker's spiro-formulae. The criticism of Noller & Ladenburg (35) that migration of the double bond "for no apparent reason" must be assumed in the anhydrosarsasapogenoic acids (XXX, XXXI) is surely unfounded since Burton & Shoppee (84) have demonstrated the facile occurrence of such prototropic changes in cyclopentenone systems.

The production of C_{22} -lactones (as XXXIII) by rather vigorous oxidation with chromium trioxide (after protection of nuclear hydroxyl groups) is characteristic of the sapogenins (14, 17, 18, 74, 75,

TABLE II

PRODUCTS DERIVED FROM SARSASAPOGENIN

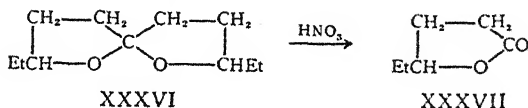
XXXIII, XXXIV, XXXV



* Formerly called in the case of sarsasapogenin "sarsasapogeninic acid" (82).

† 3(β)-Hydroxy-16-ketobisnorcholanic acid from sarsasapogenin. 3(β)-Hydroxy-16-keto-bisnorallocholanic acid from tigogenin.

85). The use of nitric acid also furnishes lactones (13, 86, 87), a simple analogy being provided by the oxidation of diethyloxetone (XXXVI) to caproic lactone (XXXVII) (88). The lactones dissolve in potassium hydroxide and are reprecipitated by acids, but are not extracted from ethereal solution by potassium hydroxide (17).



Degradation of the lactones by the Tschesche-Hagedorn procedure furnishes etiobilanic or etioallobilanic acids (as XXXV) (14, 18, 19, 20, 27). Sarsasapogenin lactone has been converted (89) into tigogenin lactone (14) confirming that the nuclei of these sapogenins differ only in configuration at C-5. The C_{22} -lactones are also obtained by reduction of the C_{22} -16-ketoacids (as XXXIV) (20, 80), but are never observed as oxidation products of the sapogenoic acids (as XXIX) (54).

If nuclear hydroxyl groups are not protected, oxidation of the sapogenins leads to fission of ring A in addition to attack on the side-chain. This complicated the early oxidative studies by Kiliani & Windaus, Fieser's interpretation of which (7) as modified by Tschesche (16) appears to be satisfactory. Thus gitogenin (XXXVIII) (and also tigogenin) gives the 2||3-gitogenic acid⁵ (XL) (16), also obtained from digitogenin (XXXIX) by chromium trioxide oxidation to digitogenic acid (the 6-keto-2||3-dibasic acid) and Wolff-Kishner reduction of this (16); further oxidation of XL gives with chromium trioxide (90) the tribasic gitogenoic acid (XLI, compare XXIX) and with nitric acid (86) the 2||3-dibasic acid lactone (XLII) (7). Oxidation of gitogenoic acid (XLI) with nitric acid gives 16-keto-bisnoralloisolithobilanic acid (XLIII) (80, 86), reduction of which affords the lactone XLII. This lactone has also been obtained by chromium trioxide oxidation of tigogenin lactone and gitogenin lactone (both as XXXIII) without protecting the hydroxyl groups, and by oxidation of tigogenone (XII) with nitric acid (80).

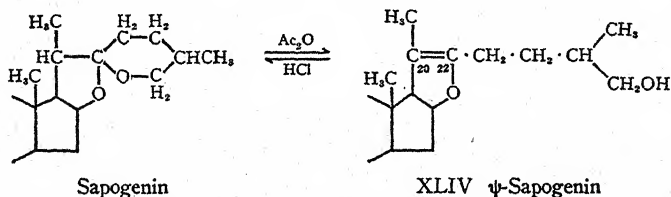
α -Methylglutaric acid (16, 91) and methylsuccinic acid (16) have been obtained by energetic oxidation of digitogenic acid; these acids undoubtedly come from the side-chain (74, 92).

⁵ The acid produced by fission of the ring system between C_x and C_y is denoted as the x||y-dicarboxylic acid.

out affecting the side-chain (101); the side-chain can also be substituted giving 5,6,23-tribromodiosgenin acetate, which can be debrominated with potassium iodide to give 23-bromodiosgenin acetate (99). Oxidation of 23-bromosarsasapogenin acetate with chromium trioxide gives the C₂₂-16-ketoacid (XXXIV); under similar conditions, 23-bromodiosgenin acetate yielded some 7-keto-23-bromodiosgenin acetate (99).

Pseudosapogenins.—The sapogenins (21, 29, 39, 70, 102, 103, 104, 110) but not their dihydro-derivatives (103) undergo a general reaction with acid anhydrides at 200° to give, after alkaline hydrolysis, good yields of isomeric products termed pseudosapogenins.⁷ Small amounts of the C₂₂-lactones (as XXXIII) are also formed. Since sarsasapogenin and smilagenin give the same ϕ -compound (103), the reaction involves fission of the six-membered oxide ring. The ϕ -sapogenins are crystalline, but give acetates which fail to crystallise [compare (70, 104)] except in the case of ϕ -diosgenin (104), which forms a 3,27-diacetate. By reduction⁸ the ϕ -sapogenins furnish crystalline dihydro- ϕ -sapogenins (34, 39, 70, 103, 104), which give crystalline diacetates⁹ also obtained by hydrogenation⁸ of the noncrystalline ϕ -sapogenin acetates (22, 39, 104). ϕ -Sarsasapogenin and its diacetate, but not dihydro- ϕ -sarsasapogenin, are attacked by ozone giving Δ^{16} -pregnene-3-ol-20-one (77).

Various formulae have been proposed for the ϕ -sapogenins; the most satisfactory is XLIV which accommodates all the observations with a single exception (see p. 122).



The ϕ -sapogenins, but not their dihydro-compounds, by brief treatment with alcoholic hydrochloric acid at 25° revert to the sapogenins (33, 70). ϕ -Sarsasapogenin (same as ϕ -smilagenin), the sole repre-

⁷ Hereafter designated ϕ -sapogenins in text and ψ -sapogenins in formula.

⁸ No observations have been recorded indicating the formation of C-20 or C-22-isomerides in these hydrogenations.

⁹ Triacetates when two nuclear hydroxyl groups are present.

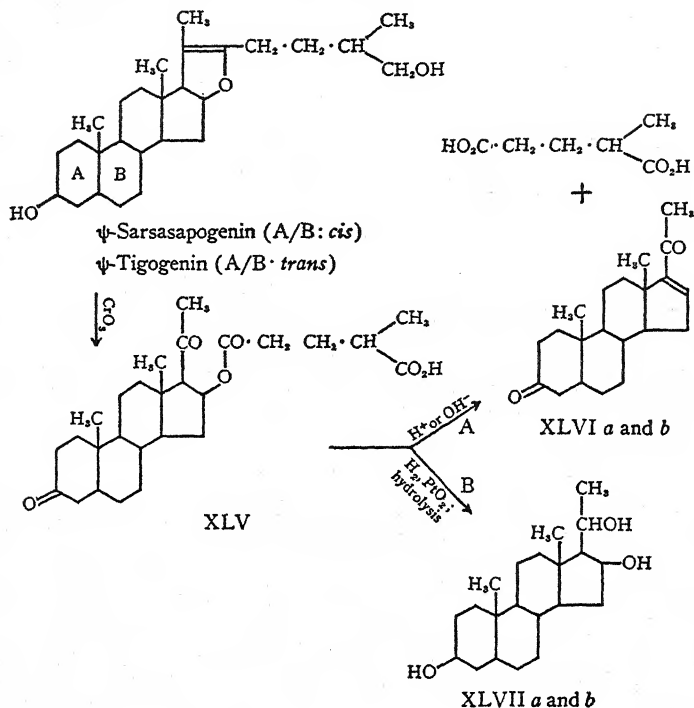
sentative of the coprostane series, reverts to sarsasapogenin (VII) and not smilagenin (XX), whilst the other φ -sapogenins belonging to the cholestane series give their parent sapogenins, all of which possess the iso-configuration at C-22 (VI). It may be noted that φ -episarsasapogenin (103) should give the known episarsasapogenin, which by prolonged treatment with hot acid should furnish the as yet unknown epismilagenin. φ -Sapogenin derivatives have also been obtained from sarsasapogenone (IX) (33), tigogenone (XII) (104), Δ^4 -tigogenone (XXI) (70), chlorogenone (XIV) (61), and 3-desoxysarsasapogenin (92).

Oxidative degradation of the φ -sapogenins and their derivatives leads to various steroid intermediates important for the preparation of androgenic, progestational, and cortical hormones, and supplies the best evidence for the structure XLIV. In early experiments, mild oxidation with chromium trioxide of φ -sarsasapogenin (21, 102, 109) and φ -tigogenin (109) gave respectively Δ^{16} -pregnene-3,20-dione (XLVIa) and Δ^{16} -allopregnene-3,20-dione¹⁰ (XLVIb), previously prepared by Butenandt, Mamoli, & Heusner (107). Repetition of these experiments (92) failed to furnish the primary oxidation products (XLV) in crystalline condition, but acid or alkaline hydrolysis of the esters (XLV) gave (route A) the Δ^{16} -pregnene- or Δ^{16} -allopregnene-3,20-diones (XLVIa and b) together with α -methylglutaric acid, whilst complete reduction followed by hydrolysis (route B) furnished pregnane- or allopregnane-3(β),16,20-triols (XLVIIa and b) respectively.

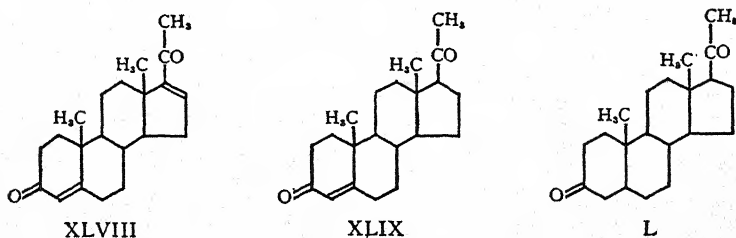
Analogous degradations have been made with φ -epitigogenin (39), φ -chlorogenin, φ -chlorogenone (61), φ -sarsasapogenone (103), and φ -tigogenone (104). φ - Δ^4 -Tigogenone (70) (which is hydrogenated to φ -sarsasapogenone using palladium-barium sulphate catalyst) furnishes $\Delta^{4,16}$ -pregnadiene-3,20-dione (XLVIII) on oxidation. The latter is hydrogenated with palladised barium sulphate catalyst, to a mixture of progesterone (XLIX) and pregnane-3,20-dione (L). The unacetylated dihydro- φ -sapogenins afford the same products (39, 61, 70). The case of dihydro- φ -sarsasapogenin (22) is considered later (see p. 122).

When the diacetate of φ -tigogenin (LI) was oxidised with chromium trioxide under mild conditions the neutral product failed to

¹⁰ The isolation of XLVIb is the first direct proof of the presence of an oxygen atom at C-3 in tigogenin.



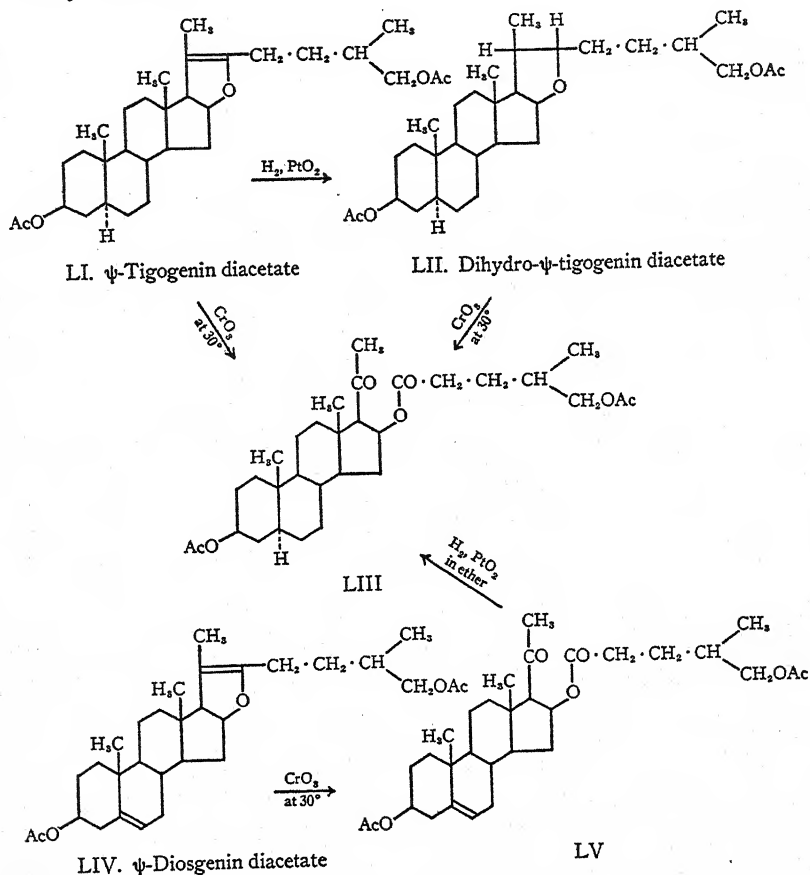
crystallise but gave by complete reduction (hydrogenation with platinum oxide catalyst in acetic acid) and hydrolysis (cf. route A) allo-pregnane-3(β),20(β^*)-diol¹¹ (106). Repetition of this experiment



and also oxidation of dihydro- ϕ -tigogenin diacetate (same as tetrahydro- ϕ -diosgenin diacetate) (LII) gave the crystalline ester LIII, also obtained from ϕ -diosgenin diacetate (LIV) by hydrogenation of

¹¹ Apart from the formation of a precipitate with digitonin, this is the first proof that the 3-hydroxyl group in tigogenin has the β -configuration.

the crystalline oxidation product LV (79). The isolation of these O_7 -esters (LIII, LV) appears to place the structure of the φ -sapogenins beyond doubt.



The ester LIII undergoes the two general degradations designated "route A" and "route B," so that the diacetates of both φ -sapogenins and dihydro- φ -sapogenins furnish identical products as shown in Table III. The triols obtained (route B) are not those formed from the sapogenins with Caro's acid, and probably differ in configuration at C-20 (92). The last column of Table III shows the diols obtained by reduction of the products obtained by route A [sodium-ethanol $\rightarrow 20(\alpha^*)$, platinum-acetic acid $\rightarrow 20(\beta^*)$].¹²

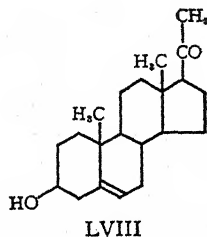
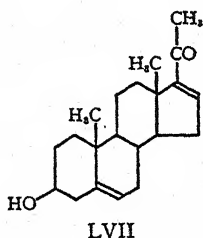
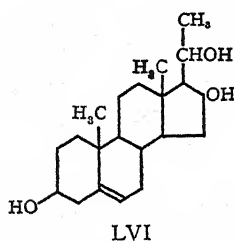
¹² See footnote 6, p. 115.

TABLE III
COMPARISON OF PRODUCTS OBTAINED FROM ψ - AND DIHYDRO- ψ -SAPOGENINS

	Reference	Route B	Route A	C
ψ -Sarsapogenin diacetate.....	(22, 23)	Pregnane-3(β),16,20(β^*)-triol (m.p. 236-240°)	Δ^{10} ,Pregnene-3(β)-ol-20-one (m.p. 207-209°)	{ Pregnane-3(β),20(α^*)-diol (m.p. 182-184°) Pregnane-3(β),20(β^*)-diol (m.p. 174-176°) }
Dihydro- ψ -sarsapogenin diacetate	(92, 108)			
ψ -Episarsapogenin diacetate...	(92, 108)	Pregnane-3(α),16,20(β^*)-triol (m.p. 203-206°)	Δ^{10} ,Pregnene-3(α)-ol-20-one (m.p. 194-196°)	{ Pregnane-3(α),20(α^*)-diol (m.p. 242-243°) Pregnane-3(α),20(β^*)-diol (m.p. 230-232°) }
Dihydro- ψ -episarsapogenin diacetate				
ψ -Tigogenin diacetate.....	(79, 92, 104)	Allopregnane-3(β),16,20(β^*)-triol (m.p. 287-289°)	Δ^{10} ,Allopregnene-3(β)-ol-20-one (m.p. 202-204°)	{ Allopregnane-3(β),20(α^*)-diol (m.p. 218°) Allopregnane-3(β),20(β^*)-diol (m.p. 196°) }
Dihydro- ψ -tigogenin-diacetate	(106, 108)			
ψ -Epitigogenin-diacetate	(39, 92)	Allopregnane-3(α),16,20(β^*)-triol (m.p. 263-265°)	Δ^{10} ,Allopregnene-3(α)-ol-20-one (m.p. 219-222°)	{ Allopregnane-3(α),20(α^*)-diol (m.p. 248°) Allopregnane-3(α),20(β^*)-diol (m.p. 207°) }
Dihydro- ψ -epitigogenin diacetate....	(108)			

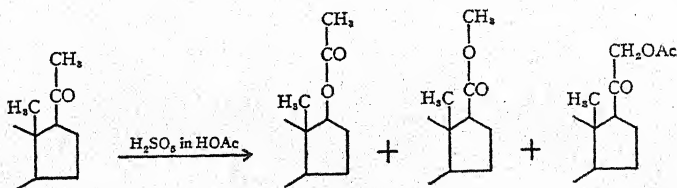
* See footnote 6, p. 115.

The Δ^5 -unsaturated ester (LV) gives (route B, using aluminium isopropylate) the unsaturated triol (LVI) (79); by route A it affords $\Delta^{5,16}$ -pregnadiene-3(β)-ol-20-one (LVII) (70), reduced by hydrogen using palladium catalyst to Δ^5 -pregnene-3(β)-ol-20-one (LVIII) (110). Oxidation of ϕ -diosgenin diacetate (LIV) as the crude dibromide with chromium trioxide also gave the compound of structure LVII after debromination, which compound is reduced by sodium-ethanol to Δ^5 -pregnene-3(β),20(α^*)-diol. Oxidation of the last named furnishes progesterone (XLIX) (70). The side-chain oxidation of ϕ -diosgenin diacetate LIV to LVII proceeds so rapidly that it can be conducted without protection of the Δ^5 -double bond (108, 110); similarly, ϕ -trillin acetate, obtained from trillin tetraacetate (same as diosgenin 3- α -glucoside tetraacetate) with acetic anhydride at 200°, can be oxidised directly, to give LVII after hydrolysis of the glucose residue at C-3 (110).

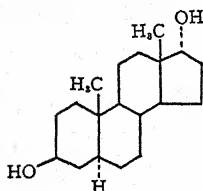


ϕ -Chlorogenin triacetate and dihydro- ϕ -chlorogenin triacetate, described (61) as a "diacetate," both yield by oxidation a noncrystalline ester, 3(β),6(α),27-triacetate, analogous to LIII, which is convertible into allopregnane-3(β),6(α)-diol-20-one. 3-Desoxy- ϕ -sarsasapogenin acetate and its dihydroderivative give by route A Δ^{16} -pregnene-20-one, which may be hydrogenated with palladium catalyst to pregnane-20-one (92).

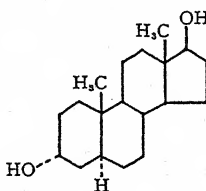
20-Keto-steroids undergo the apparently general reaction of Baeyer & Villiger with Caro's acid:



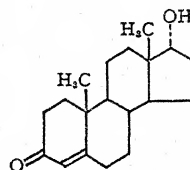
whereby allopregnane-20-one gives androstane-17(α)-ol acetate, etioallocholanolic acid, and allopregnane-21-ol-20-one acetate (105, 109); and pregnane-3(α)-ol-20-one acetate similarly affords etiocholane-3(α),17(α)-diol diacetate and etiolithocholic acid (104, 105, 109). The reaction has been used to convert the acetate of allopregnane-3(β)-ol-20-one, obtained by catalytic hydrogenation of Δ^{16} -allopregnene-3(β)-ol-20-one (see Table III) using palladium as catalyst, to androstane-3(β),17(α)-diol (dihydroisoandrosterone) (LIX); 3(β)-hydroxyetioallocholanolic acid and the diacetate of allopregnane-3(β),21-diol-20-one, previously prepared by Reichstein & von Euw (111) were also obtained (104, 109). The acetate of Δ^{16} -allopregnene-3(α)-ol-20-one (see Table III) has similarly been converted to androstane-3(α),17(α)-diol (dihydroandrosterone) (LX) (39). In both these hormone preparations the starting material was diosgenin. The method has also been used to obtain testosterone (LXI) (105): Δ^{16} -pregnene-3,20-dione (XLVIa) was reduced with palladium to pregnane-3,20-dione, which by oxidation as the 4-bromodiketone with Caro's acid gave, after elimination of hydrogen bromide with pyridine, testosterone acetate plus desoxycorticosterone (LXII) (not isolated as such). Compounds containing acetoxy or hydroxyl groups at C-3 and also α -bromo substituted steroid 3-ketones can be used for the oxidations without affecting these groups (105).



LIX



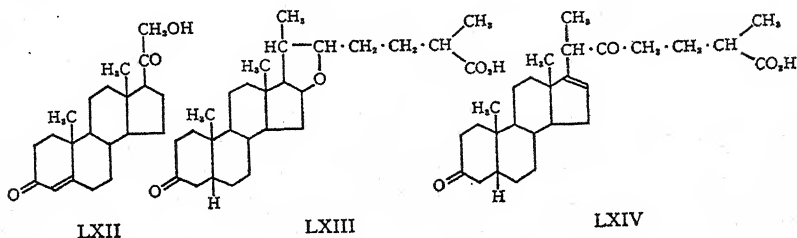
LX



LXI

The only observation not satisfactorily accounted for in terms of the ϕ -sapogenin structure XLIV relates to the dihydro- ϕ -sapogenins (as LII) which should be identical or at least stereoisomeric with the dihydrosapogenins (79). Dihydrosarsasapogenin (XXIV) gives by mild oxidation with chromium trioxide a C_{27} -monoketo acid (LXIII) unaccompanied by Δ^{16} -pregnene-3,20-dione (XLVIa) (34, 77), whilst under the same conditions dihydro- ϕ -sarsasapogenin (compare LII) is said to give XLVIa together with a C_{27} -diketo acid,

which is stated (22) to have the formula $C_{27}H_{42}O_4$, although it is given (22, 77) the structure LXIV which is $C_{27}H_{40}O_4$. This acid is said to give a disemicarbazone, and by further oxidation to yield XLVIa (22, 77), observations which are incompatible with the formula $C_{27}H_{42}O_4$.

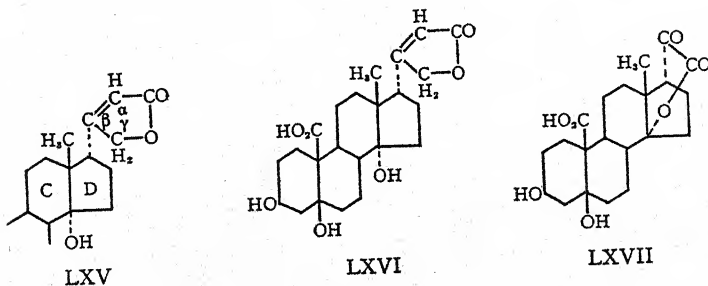


CARDIAC GLYCOSIDES

DIGITALIS-STROPHANTHUS GROUP

The important paper by Dimroth & Jonsson (10) indicates that rings C and D of the natural steroids possess the *trans*-configuration; by implication the cardiac glycosides and aglycones should be formulated as in LXV.

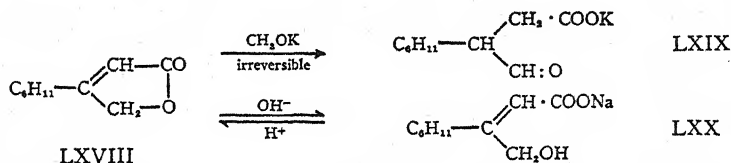
Two independent investigations indicate that the characteristic and physiologically important unsaturated γ -lactone ring possesses the $\Delta^{\alpha,\beta}$ -structure (LXV). The oxidation of strophanthidinic acid (LXVI) to (LXVII) (112), which was inexplicable on the basis of $\Delta^{\beta,\gamma}$ -structure hitherto assigned, is now readily accounted for.



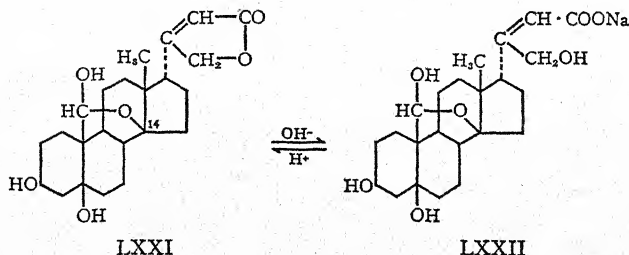
Elderfield *et al.* (113, 114, 115) have synthesised model β -substituted $\Delta^{\alpha,\beta}$ -butenolides; in contrast to the reported behaviour of $\Delta^{\alpha,\beta}$ -angelica lactone (116), β -cyclohexyl- $\Delta^{\alpha,\beta}$ -butenolide (LXVIII) (117)

resembles strophanthidin in (a) the Tollens & Legal test (improved modification with potassium ferricyanide), (b) the ultraviolet absorption spectrum (maximum at 220 m μ), (c) the Zerewitinoff determination (production of 0.43 mol. methane), and (d) hydrogenation with Adams catalyst, by which β -cyclohexylbutyrolactone is formed unaccompanied by acidic products. This behaviour (d) parallels that of the natural aglycones and contrasts with that of $\Delta^{\beta,\gamma}$ -lactones, which furnish varying amounts of desoxyacids (118).

The lactone (LXVIII) exhibits two distinct modes of reaction with alkali (117):

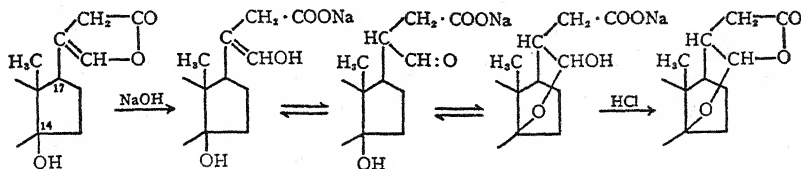


The exact mechanism of production of LXIX is obscure, but probably involves the primary change $\Delta^{\alpha,\beta}$ -lactone \rightarrow $\Delta^{\beta,\gamma}$ -lactone, which is shown to be irreversible. The compound LXIX is also formed together with the substance LXX in aqueous-alcoholic solution. At 25° only traces of compound LXIX result, but at the boiling point of the medium substance LXIX forms two thirds of the product. The reversibility of the change LXVIII \rightleftharpoons LXIX accords well with the ready relactonisation of φ -strophanthidin¹³ (LXXI) (116) after saponification:

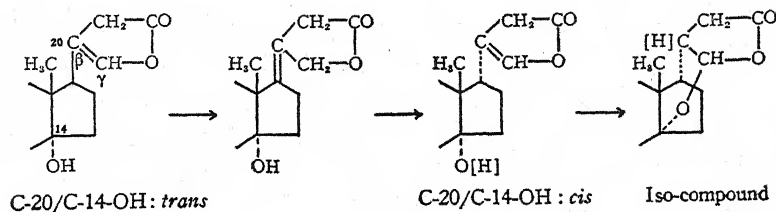


¹³ The formation of LXXI from strophanthidin (LXXIV) with concentrated hydrochloric acid appears to involve inversion of the natural configuration at C-14 to permit bridge formation with the C-10 aldehyde group.

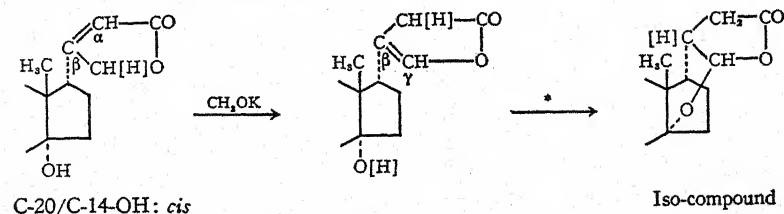
The usually accepted mechanism of the general and irreversible formation of isocompounds (119):



is not in harmony with the experimental observation (120) that, in the formation of isostrophanthidin from strophanthidin with methyl alcoholic potassium hydroxide, preliminary saponification of the lactone ring is not necessary. The interpretation¹⁴ advanced by Jacobs & Elderfield (121) to account for this fact:

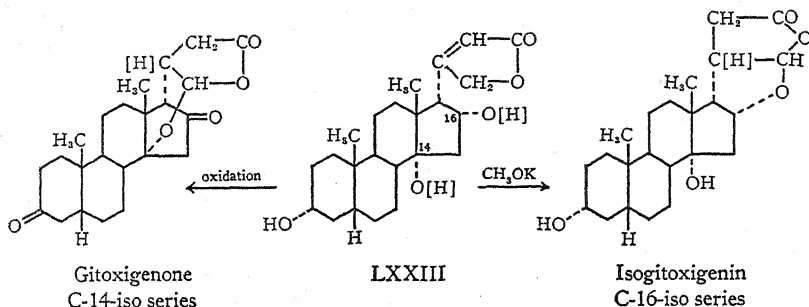


can now be replaced by the simple sequence:

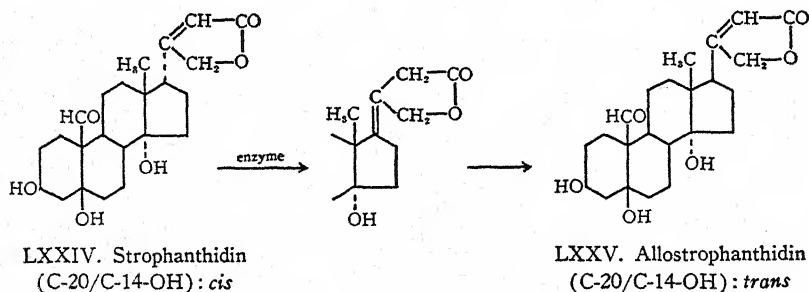


The case of gitoxigenin (LXXIII), which furnishes two different types of iso-compound, may be represented as follows, and it appears that the hydroxyl group on C-16 must possess the α -configuration, i.e. (C-13-Me/C-16-OH) *trans*:

¹⁴ In the version given by Elderfield *et al.* (117) their formulae VI and VIII should be interchanged.



If the existing configuration (C-20/C-14-OH) is *trans*, spatial factors preclude the occurrence of the intramolecular addition reaction (*), and prevent the formation of an iso-compound. This is probably the case in allocymarin (122) and alloemicymarin (123, 124). These alloglycosides are formed from the natural glycosides by the action of an enzyme present in the plant (122); since the sugar component is unaltered, the isomerism relates to the aglycone, which according to Tschesche & Bohle¹⁵ (125) undergoes inversion at C-17



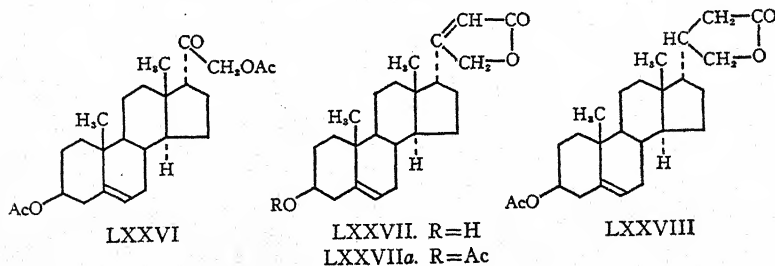
because iso-compounds can no longer be obtained (122, 125). The alloglycosides and aglycones are physiologically inactive,¹⁶ although

¹⁵ The formulae given by Tschesche & Bohle for strophanthidin and allostrophanthidin should be interchanged: in the version given above the lactone rings are shown correctly as $\Delta^{\alpha,\beta}$ -unsaturated.

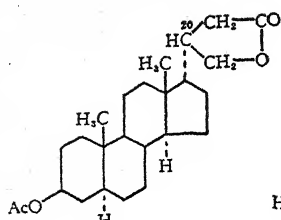
¹⁶ It may be noted that *cistestosterone* (whose configuration at C-17 corresponds with that of (LXXV), 17-isoprogerone, and 17-isodesoxycorticosterone are all less biologically active than their 17-isomerides.

retaining all the functional groups of the active isomerides and the $\Delta^{\alpha,\beta}$ -unsaturated lactone ring [LXXV gives the Legal test (122)]. The isomerism is maintained after removal of all three hydroxyl groups from emicymarigenin and alloemicymarigenin (123, 124). Similarly, Bloch & Elderfield (126) have shown that isomerism between LXXIV and LXXV persists after removal of the asymmetry centres at C-3 and C-5, and they consider that the isomerism must arise by inversion at C-14 or C-17, preferring the former possibility on the ground that a ϕ -allostrophanthidin analogous to ϕ -strophanthidin (LXXI) could not be prepared. This preference appears untenable to the reviewer since (a) the conversion of strophanthidin (LXXIV) into ϕ -strophanthidin (LXXI) must now be regarded as involving inversion of the natural configuration at C-14 (see footnote 13), and (b) the isomerism survives removal of the asymmetric centre at C-14, because the ethyl semiacetals of dianhydrostrophanthidin (127) and of dianhydroallostrophanthidin (122) are different.¹⁷

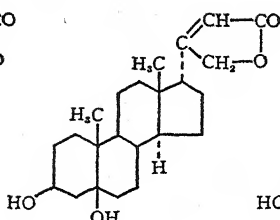
The second line of evidence is as follows: Ruzicka, Reichstein *et al.* (129, 130) have applied the Reformatsky reaction to 20-keto-steroids. From 3,21-diacetoxy- Δ^5 -pregnene-20-one (LXXVI) and ethyl bromoacetate the lactones LXXVII and LXXVIIa were isolated. The product LXXVII, m.p. 262°, gives an immediate positive Legal test, and also the ultraviolet absorption spectrum of a $\Delta^{\alpha,\beta}$ -unsaturated lactone, and appears to be identical with α -anhydrouzarigenin, m.p. 263–265° (131). With Raney nickel catalyst in alcohol at 20° the compound LXXVIIa absorbs exactly 1 mole of hydrogen to form the substance LXXVIII



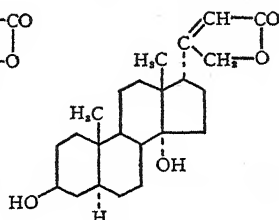
¹⁷ Since these dianhydrosemiacetals were prepared under the same conditions, the two double bonds introduced might reasonably be expected ultimately to occupy the same positions, irrespective of the original direction of dehydration (128).



LXXIX



LXXX



LXXXI

which no longer gives the Legal test. Hydrogenation of the compound LXXVIIa using platinum oxide catalyst in acetic acid, affords an acetoxytetrahydrolactone (LXXIX) which appears to be identical with α_2 -tetrahydroanhydrouzarigenin acetate: similar hydrogenation of the substance LXXVII followed by acetylation furnishes a product (LXXIX) apparently identical with α_1 -tetrahydroanhydrouzarigenin acetate, together with the α_2 -isomeride [cf. Tschesche (131)]. The physical properties of the C-20-isomerides (LXXIX) and of the α_1 - and α_2 -tetrahydroanhydrouzarigenin acetates are set out in Table IV.

TABLE IV

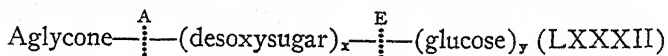
	Ruzicka <i>et al.</i>		Tschesche	
	Melting point °C	Optical rotation [α] _D (CHCl ₃)	Melting point °C	Optical rotation [α] _D (CHCl ₃)
LXXIX α_1	243	+5.9°	248	+3.9°
LXXIX α_2	203	+19.7°	205	+20.2°

It is pointed out (130) that the nuclear structure LXXX for uzarigenin originally suggested by Tschesche (132) must be reconsidered. A revision was later made by Tschesche in which the tertiary hydroxyl group was moved to C-14 (LXXXI) because uzarin underwent an inexactly explained change with alkali (133, 134). It is suggested that this explanation is insufficient since substance LXXVII is also altered by alkaline reagents.

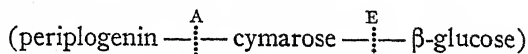
It follows that the singular position of uzarigenin as the only aglycone with the A/B-*trans* configuration becomes improbable. The lack of physiological activity displayed by uzarin may arise from the absence of a hydroxyl group on C-14; the alternative explanation that uzarin is an alloglycoside appears to be excluded since by degradation

Tschesche (131) obtained etioallocholanolic acid and not a corresponding 17-iso acid (135).

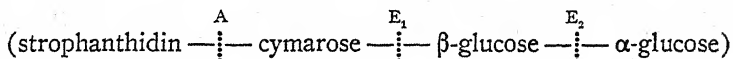
Stoll & Renz (136) formulate the genuine cardiac glycosides as LXXXII, the sugar residue being attached, according to Tschesche (160) always to the hydroxyl group at C-3 of the aglycone.



Acetic acid may also be present in a sugar acetyl group as in the diglilanides A, B, and C (137) which are easily hydrolysed by lime water to the desacetyldigilanides A, B, and C (138). Fission at E is caused by enzymes occurring in the plant, fission at A by acid or alkaline hydrolysis. Thus periplocin



is hydrolysed with strophanthobiase (139) at (E) to give periplocymarin + glucose; it is hydrolysed with 0.1 *N*-acid at (A) to form periplogenin + periplobiose (136). Similarly *k*-strophanthoside



when split with α -glucosidase at (E₂) yields glucose + *k*-strophanthin- β , the latter being hydrolysed by 0.1 *N*-acid to the aglycone + strophanthobiose (136); splitting with strophanthobiase at (E₁) yields cymarin + 2 mol. glucose;¹⁸ and finally hydrolysis at (A) with 0.1 *N*-acid produces the aglycone + strophanthotriose (140, 141). When, as sometimes happens, the carbohydrate moiety directly attached to the aglycone is not a desoxysugar, hydrolysis can only be effected under conditions so drastic as to cause alteration of the aglycone, and alcoholysis has been used (142).

A list of natural cardiac glycosides, their physiological activities and their hydrolysis products, is given in Table V. The more recent literature is also cited.

The anhydro-compounds of uzarigenin, thevetigenin, and cerberigenin furnish insoluble digitonides, and the hydroxyl groups on C-3 of these aglycones probably have the β -configuration. Digitoxigenin and digoxigenin are known to be 3(α)-hydroxy-compounds. Strophanthidin, allostrophanthidin, anhydrosarmentogenin, gitoxigenin, and

¹⁸ Strophanthobiase (from *S. courmontii* seeds) here acts as an α -glucosidase.

TABLE V*
CARDIAC GLYCOSIDES AND FISSION PRODUCTS

Glycoside	Mean lethal dose for cats $\mu\text{g./kg.}$	Formula	Aglycone or anhydroaglycone	Formula	Melting point $^{\circ}\text{C.}$	Position of hydroxyl groups†	Configuration of rings A/B†	Sugar
<i>k</i> -Strophanthoside (140)...	126 ^a , 1159 ^f	$\text{C}_{49}\text{H}_{84}\text{O}_{10}$	Strophanthidin	$\text{C}_{23}\text{H}_{32}\text{O}_6$	136 & 235	3,5,14§	<i>cis</i>	$\left\{ \begin{array}{l} \text{Cymarose} + 2 \text{ Glucose} \\ \text{Cymarose} + \text{Glucose} \\ \text{Cymarose} (\text{C}_7\text{H}_{14}\text{O}_5) \end{array} \right.$
<i>k</i> -Strophanthin- β	160 ^a , 120 ^a	$\text{C}_{37}\text{H}_{64}\text{O}_{14}$		$\text{C}_{23}\text{H}_{32}\text{O}_6$				
Cymarin (169)	130 ^a , 126 ^a , 130 ^b , 110 ^b , 95 ^c , 111 ^a	$\text{C}_{30}\text{H}_{44}\text{O}_9$		$\text{C}_{23}\text{H}_{32}\text{O}_6$				
	Inactive	$\text{C}_{30}\text{H}_{44}\text{O}_9$		$\text{C}_{23}\text{H}_{32}\text{O}_6$				
Allocymarin (122, 124)...	120 ^f	$\text{C}_{30}\text{H}_{46}\text{O}_9$	Allostrophanthidin (124)	$\text{C}_{23}\text{H}_{32}\text{O}_6$	185	3,5,14§	<i>cis</i> ?	Cymarose
Periplocin (136)	151 ^a , 156 ^b , 135 ^f	$\text{C}_{33}\text{H}_{44}\text{O}_{10}$	Periplogenin	$\text{C}_{23}\text{H}_{32}\text{O}_6$		3,5,14	<i>cis</i>	$\left\{ \begin{array}{l} \text{Cymarose} + \text{Glucose} \\ \text{Cymarose} \end{array} \right.$
Periplocymarin		$\text{C}_{33}\text{H}_{44}\text{O}_{10}$						
Emicymarin (124)	170 ^b , 137 ^b	$\text{C}_{30}\text{H}_{46}\text{O}_9$	Trianhydroperiplogenin	$\text{C}_{23}\text{H}_{32}\text{O}_6$	192	3,5,14		Digitulose ($\text{C}_7\text{H}_{14}\text{O}_5$)
Alloemicymarin (123, 124)	$\text{C}_{30}\text{H}_{46}\text{O}_9$	Trianhydroallopriperigenin	$\text{C}_{23}\text{H}_{32}\text{O}_6$	154	3,5,14		Digitulose
Sarmentocymarin	210 ^b	$\text{C}_{30}\text{H}_{46}\text{O}_9$	Sarmentogenin (143)	$\text{C}_{23}\text{H}_{32}\text{O}_6$	270	3,14?	<i>cis</i>	$\left\{ \begin{array}{l} \text{Sarmentose} (\text{C}_7\text{H}_{14}\text{O}_4) \\ 3 \text{ Digitoxose} + \text{Glucose} \\ + \text{Acetic acid} \end{array} \right.$
Digitanide A	320 ^f , 380 ^a	$\text{C}_{49}\text{H}_{86}\text{O}_{10}$						
Desacetyldigitanide A	369 ^a	$\text{C}_{47}\text{H}_{74}\text{O}_{10}$	Digitoxigenin	$\text{C}_{23}\text{H}_{34}\text{O}_4$	250	3(α), 14	<i>cis</i>	$\left\{ \begin{array}{l} 3 \text{ Digitoxose} + \text{Glucose} \\ 3 \text{ Digitoxose} + \text{Glucose} \\ 3 \text{ Digitoxose} (\text{C}_6\text{H}_{12}\text{O}_5) \end{array} \right.$
Digitoxin	330 ^a , 327 ^d , 420 ^a	$\text{C}_{41}\text{H}_{64}\text{O}_{13}$						
Somalin (144)	$\text{C}_{30}\text{H}_{46}\text{O}_9$	Adynerigenin Anhydrothevetigenin	$\text{C}_{23}\text{H}_{32}\text{O}_6$	242	3(α), 14	<i>cis</i>	Cymarose
Adynerin (145, 146, 147)	Inactive	$\text{C}_{30}\text{H}_{44}\text{O}_9$		$\text{C}_{23}\text{H}_{32}\text{O}_6$	220	3(β), 14	<i>cis</i>	Oleandrose ($\text{C}_7\text{H}_{14}\text{O}_4$)
Thevetin (148, 149)	920 ^a	$\text{C}_{44}\text{H}_{66}\text{O}_{15}$						Digitulose + 2 Glucose
Digitanide B	346 ^f , 400 ^f , 403 ^a	$\text{C}_{49}\text{H}_{86}\text{O}_{10}$						$\left\{ \begin{array}{l} 3 \text{ Digitoxose} + \text{Glucose} \\ + \text{Acetic acid} \end{array} \right.$
Desacetyldigitanide B	369 ^a	$\text{C}_{47}\text{H}_{74}\text{O}_{10}$	Gitoxigenin	$\text{C}_{23}\text{H}_{34}\text{O}_6$	235	3,14,16	<i>cis</i>	$\left\{ \begin{array}{l} 3 \text{ Digitoxose} + \text{Glucose} \\ 3 \text{ Digitoxose} \end{array} \right.$
Gitoxin	750 ^f , 727 ^a	$\text{C}_{41}\text{H}_{64}\text{O}_{14}$						2 Digitoxose
Gitalin	$\text{C}_{32}\text{H}_{50}\text{O}_{11}$						Digitulose + Glucose
Digitalin	$\text{C}_{30}\text{H}_{46}\text{O}_9$						Digitulose + 2,6-Desoxyhexose ($\text{C}_6\text{H}_{12}\text{O}_4$)
Oridigin (150)	$\text{C}_{33}\text{H}_{50}\text{O}_{13}$	Dianhydrodigitoxigenin (Digitalligenin)	$\text{C}_{23}\text{H}_{30}\text{O}_8$	213	3,14,16	<i>cis</i>	

TABLE V—(Concluded)

Glycoside	Mean lethal dose for cats μg./kg.	Formula	Aglycone or anhydroaglycone	Formula	Melting point °C.	Position of hydroxyl groups†	Configuration of rings A/B†	Sugar
Oleandrin (146, 151, 152)	200 ^b , 240 ^f	$C_{25}H_{45}O_9$	Oleandrigenin	$C_{25}H_{45}O_6$	223	3,14,16††	cis	Oleandrose
Desacetyloleandrin (146)	308 ^e	$C_{26}H_{47}O_8$	Neriantogenin	$C_{25}H_{43}O_4$	259	3,16	cis	Glucose
Nerianin (147)	Inactive	$C_{26}H_{47}O_8$						{ 3 Digitoxose + Glucose + Acetic acid
Digitanide C.	230 ^a , 280 ^a	$C_{49}H_{76}O_{20}$					cis	{ 3 Digitoxose + Acetic acid
α- & β-Acetyldigoxin (150)	$C_{43}H_{66}O_{15}$	Digoxigenin (153, 154, 155, 156, 157)	$C_{23}H_{34}O_5$	222	3(a),12,14	cis	{ 3 Digitoxose
Digoxin	220 ^a , 225 ^d , 424 ^f , 280 ^a	$C_{41}H_{64}O_{14}$			263 [†]		?	
Izarin	5080 ^a	$C_{30}H_{44}O_{14}$	{ α-Anhydrouzarigenin (129, 130)	$C_{23}H_{32}O_3$	237 [†]	3(β),5	cis?	2 Glucose
Convallatoxin (158)	80 ^a , 74 ^b	$C_{29}H_{42}O_{10}$	{ β-Anhydrouzarigenin	$C_{23}H_{30}O_3$		3,5,8,14**	cis?	Rhamnose
Ouabatin (159, 160)	120 ^a , 86 ^b , 95 ⁿ	$C_{29}H_{44}O_{12}$	Anhydroconvallatoxin (Ouabagenin)	$C_{23}H_{34}O_8$		3,10,14,?,?,?	?	Rhamnose
β-Antiarin (161)	100 ^a , 94 ^b	$C_{29}H_{42}O_{11}$	Dianhydroantiarigenin	$C_{23}H_{32}O_5$	167	3,5,14,?,††	?	{ Rhamnose
α-Antiarin (161)	130 ^b , 96 ^b	$C_{28}H_{40}O_{11}$			222	3(β),14	cis?	{ Antiarose ($C_6H_{12}O_5$)
Cerberin (162)	147 ^m	$C_{28}H_{44}O_8$	Anhydrocerberigenin	$C_{23}H_{32}O_3$	115	3		{ Cerberose ($C_6H_{12}O_5$)
Digmin (163, 164)	14725 ^p	$C_{28}H_{40}O_7$	Digminigenin	$C_{23}H_{35}O_4$	221	3,14,16,?,?		{ Diginose ($C_6H_{14}O_4$)
Calotropin (165, 166)	120 ^b , 103 ^c	$C_{29}H_{40}O_9$	Anhydrocalotropagenin	$C_{23}H_{32}O_6$				{ Methylreductive acid ($C_6H_8O_3$)
Uscharidin (165, 166)	$C_{26}H_{40}O_9$	Isoanhydrocalotropagenin	$C_{23}H_{32}O_6$	251	3,14,16,?,?		{ Hydroxymethylreductive acid ($C_6H_8O_3$)
Calotoxin (165, 166)	111 ^k	$C_{26}H_{40}O_{10}$	ψ-Anhydrocalotropagenin	$C_{23}H_{32}O_6$	242	3,14,16,?,?		

* For references prior to 1936 see Fieser (7).

† Of original genin.

‡ Letters indicate bibliographical references:

a = (226)

b = (227)

c = (228)

d = (229)

e = (230)

f = (231)

g = (167)

h = (232)

i = (233)

j = (234)

k = (235)

l = (236)

m = (237)

n = (238)

p = (239)

§ -CH:O at C-10.

|| Double bond, Δ^{9,10}.

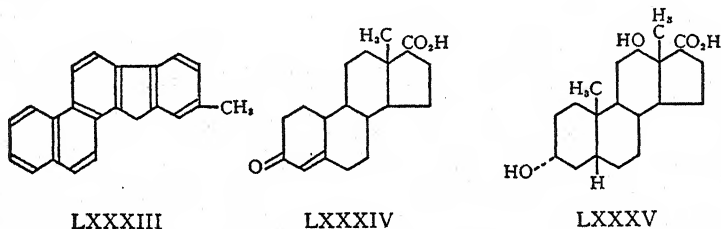
†† Hydroxyl group at C16 acetylated.

** Double bond, Δ^{9,11}.

†† -CH:O at C-10.

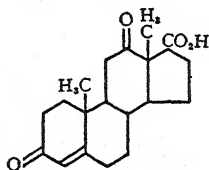
adynerin fail to give insoluble digitonides, and may be 3(α)-hydroxy-compounds, although the ready production of 3:10-oxide bridges in the two first-named compounds (122, 127) suggests that they possess the β -configuration. The excellent accounts of the chemistry of the cardiac aglycones already available (6, 7, 167) may be supplemented by the following summary of subsequent work.

Strophanthidin.—The abnormal selenium dehydrogenation (168) of the aglycone [which possesses a double melting point (169)] has been re-examined (170), and the hydrocarbon which was thought to be $C_{21}H_{16}$ (168) has been shown to be 7-methyl-2',1'-naphtha-1,2-fluorene (LXXXIII), $C_{22}H_{16}$, m.p. 301° (171). The aglycone has also been converted into an estrane derivative (LXXXIV) which is devoid of estrogenic properties (172).

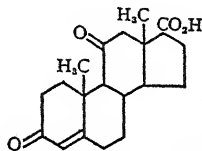


Digoxigenin.—Tschesche & Bohle (153) suggested that the hydroxyl groups in digoxigenin are at C-3, C-11, and C-14. Steiger & Reichstein (155) degraded digoxigenin via the dihydroxyetiocholenic acid (LXXXV) to a diketioetiocholenic acid (LXXXVI) different from that (LXXXVII) obtained from corticosterone. Mason & Hoehn (156) found compound LXXXVI to be identical with the diketioetiocholenic acid from desoxycholic acid but that the substance LXXXV differed from etiodesoxycholic acid; later (157), 12-epi-etiodesoxycholic acid was prepared and shown to be identical with substance LXXXV. Digoxigenin (LXXXVIII) is therefore a 3(α),-12,14-trihydroxy compound, in which the hydroxyl group on C-12 has the configuration opposite to that of the corresponding hydroxyl group in desoxycholic acid.

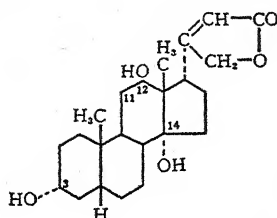
Sarmentogenin.—Tschesche & Bohle (143) converted sarmentogenin into a dehydrolactone also obtained (153) from digoxigenin. They regarded these aglycones as isomeric 3(α),11,14-trihydroxy compounds and referred the isomerism to a *cis*-B/C-ring-union in sarmentogenin. Since digoxigenin actually has structure LXXXVIII,



LXXXVI



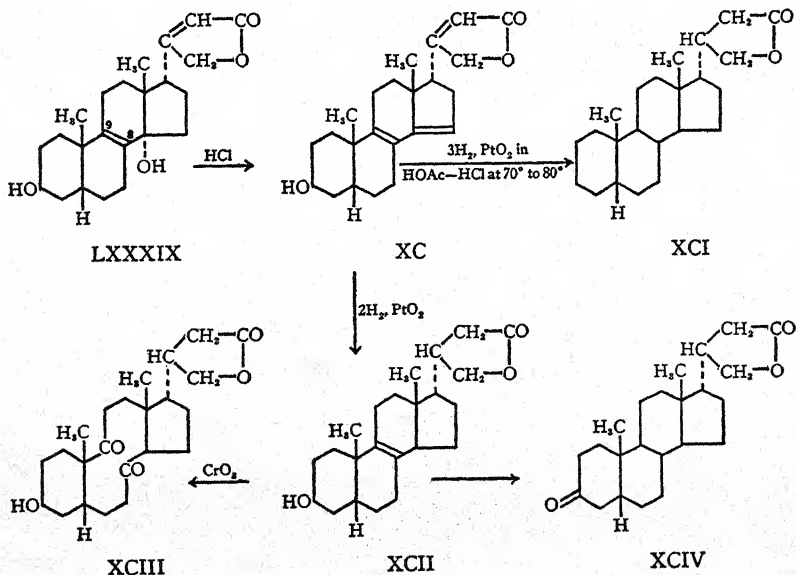
LXXXVII



LXXXVIII

this unique assumption is no longer necessary. Sarmentogenin may be 12-epidigoxigenin, or alternatively a 3(α),11,14-trihydroxy compound since hydroxyl groups at C-11 but not at C-12 are readily eliminated (173, 174, compare 143).

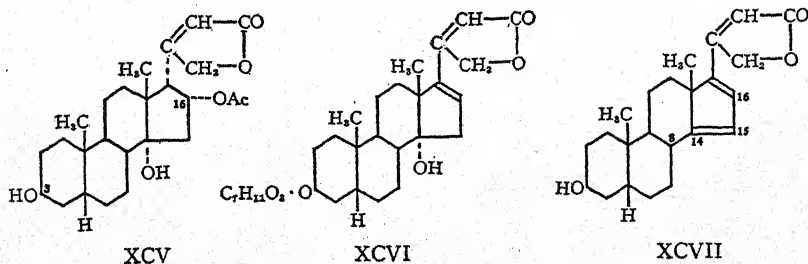
Adynerigenin.—This aglycone (LXXXIX) from the glycoside adynerin (*ex* oleander leaves) (145, 146) contains two double bonds, one of which is very difficult to reduce; it contains one secondary hydroxyl group (monoacetate formed) and has a tertiary hydroxyl group at C-14 since it affords an isoaglycone and a monoanhydro-compound (XC). The latter gives the Legal test and an ultraviolet absorption spectrum which indicates that the new double bond is conjugated with the difficultly reducible nuclear double bond.



Reduction of the monoanhydro compound XC gives the lactone XCII, oxidisable to an O_5 -compound probably XCIII, whilst forced reduction of XC gives the saturated lactone XCI from digitoxigenin, the hydroxyl group on C-3 being eliminated in the process (147). The position of the secondary hydroxyl at C-3 is proved (147) by hydrogenation of the acetate of XCII when the 3-acetoxy group survives to yield, after hydrolysis and oxidation, tetrahydroanhydrodigitoxigenone (XCIV).

Oridigin, digorid A and B.—These glycosides have been isolated from *Digitalis orientalis* (150); oridigin consists of gitoxigenin united to an unidentified 2,6-desoxyhexose and glucose. Digorid A and B are identical respectively with β - and α -acetyldigoxin, first obtained by Stoll & Kreis (175) by partial hydrolysis of the original glycoside digilanide C from *Digitalis lanata*; the isomerism concerns the acetyl group in the trisaccharide portion of the molecule, the α -compound being partially converted by hot alcohol into the more stable β -isomere (150).

Oleandrigenin.—The glycoside oleandrin (176) [same as folinerin (177)] with hot normal acid gives dianhydrogitoxigenin (XCVII) (146, 152); use of 0.02*N* sodium hydroxide (146) or 0.05*N* hydrochloric acid (151) gives oleandrigenin (XCV), which has been proved to be 16-acetoxylgitoxigenin by hydrolysis to and preparation from gitoxigenin (146). The same results were obtained (152) by vacuum pyrolysis of the glycoside and the aglycone which give respectively desacetylanhydrooleandrin (XCVI) and dianhydrogitoxigenin (XCVII). The former compound (XCVI) is converted into the latter (XCVII) with hot 2*N* acid which indicates that structure XCVII



is correct rather than the $\Delta^{8:14,15:16}$ -structure suggested by Fieser (7). The position of the acetoxy group is proved (151) by oxidation of the substance XCV to the 3-ketone, oleandrigenone, which is converted

to dianhydrogitoxigenone (compound XCVII with a keto group at C-3). Oleandrin is the first digitalis glycoside to be found which is also a steroid acetate.

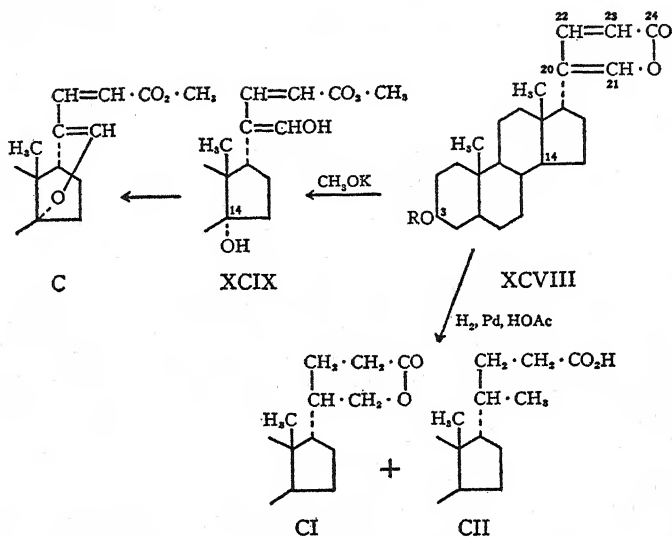
Cerberigenin.—Cerberigenin appears to resemble closely thevetigenin, although derivatives of the two aglycones are stated to be different. The glycoside gives the Legal test, and affords an isoglycoside; the hydroxyl group on C-14 so indicated is eliminated during hydrolysis, and reduction of the anhydrocerberigenin so formed, followed by oxidation of the 3-hydroxyl group, gives a saturated 3-ketolactone, different from that (XCIV) from digitoxigenin (147, 178) and the isomerides from uzarigenin (131).

Diginin.—Diginin (163) differs from all other *Digitalis* glycosides by furnishing an aglycone $C_{21}H_{28}O_4$ (164). It has no lactone ring although it gives the Legal test. It possesses no biological activity (163). Of the four oxygen atoms, one occurs as a secondary hydroxyl group, one as an aldehyde group, one as a hindered carbonyl group. The last oxygen is inert and possibly possesses an oxidic function (164).

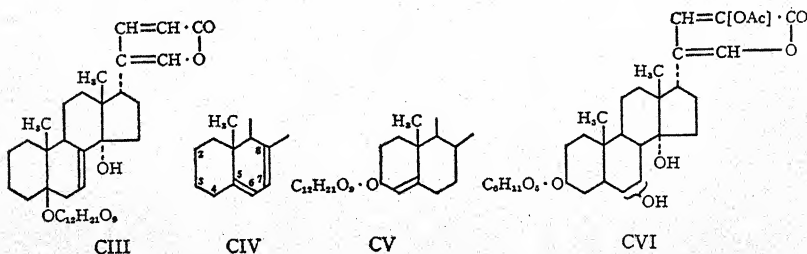
Calotropins.—The calotropins (165, 166) contain, instead of the usual sugar residue, reductinic acids, one enolic hydroxyl group of which is free and the other forms an ether with a nuclear hydroxyl group. Of the six oxygen atoms in anhydrocalotropagenin, two occur in the unsaturated lactone ring, two are present as nuclear hydroxyl groups which react with the lactone ring to give two series of isocompounds, and two are unclassified.

SQUILL GROUP; TOAD POISONS

The squill glycosides and the bufotoxins possess the general structure XCVIII; the physiologically important doubly unsaturated δ -lactone ring is characterised by a band in the ultraviolet spectrum with a maximum at 300 m μ (179), and undergoes fission with alcoholic potassium hydroxide to give enol-esters (XCIX) which give oxido-esters (isocompounds) (C) by dehydration if a suitably orientated hydroxyl group is present on C-14. By contrast with the $\Delta^{\alpha,\beta}$ - γ -lactones of the digitalis-strophanthus group, the δ -lactones (XCVIII) by reduction furnish saturated lactones (CI) invariably accompanied by considerable yields of cholanolic acids (CII). In the case of scillaridin A, the cholanolic acid formed (CII) was identified as allocholanolic acid (180, 181).



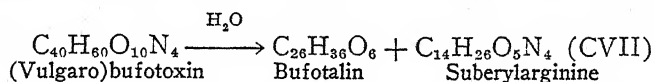
Scillaren A, scillaridin A.—Scillaren A [m.p. 270°; $\text{C}_{38}\text{H}_{52}\text{O}_{13}$; cat unit ($\mu\text{g.}/\text{kg.}$), 150(226), 145(238)], is hydrolysed by the specific enzyme scillarenase (which occurs in the plant) to proscillaridin A, m.p. 213°, $\text{C}_{30}\text{H}_{42}\text{O}_8$, and glucose; hydrolysis with 0.2*N* acid suffices to give the monoanhydro-aglycone scillaridin A [m.p. 250°; $\text{C}_{24}\text{H}_{30}\text{O}_8$; cat unit ($\mu\text{g.}/\text{kg.}$) > 6000 (238)], and rhamnose plus glucose. Stoll therefore formulated scillaren A as CIII and scillaridin A as CIV (182). Tschesche (160) and Fieser (7) suggested that the biose residue in scillaren A should be placed at C-3 (CV), and Stoll (183) has now proved this allocation to be correct by transforming the glycoside into 3(β)-hydroxyallocholanolic acid. Scillaridin A must be regarded as the 2,4- or 3,5-diene analogous to CIV.



Scilliroside.—This glucoside, $\text{C}_{32}\text{H}_{46}\text{O}_{12}$, from the red squill is distinguished by its toxicity to rats, and is of interest as possessing

an acetoxy group on the δ -lactone ring. The ultraviolet absorption spectrum is exactly like that given by proscillaridin A; the glucoside resists hydrolysis, furnishes an isocompound, and Stoll has provisionally assigned formula CVI (184).

In the bufotoxins, the hydroxylated structure XCVIII is conjugated with suberylarginine, possibly through the 3-hydroxyl group. A characteristic feature also is the presence of steroid acetyl groups which appear indispensable for physiological activity (185). In the case of the (vulgaro)bufotoxin of Wieland, hydrolysis affords the steroid moiety termed¹⁹ a bufogenin (German usage) or bufagin (American usage) (186),



but more generally an anhydro-compound results. The bufagins together with related compounds occur with the bufotoxins in the venom, and are probably formed from the latter by enzyme action. Table VI shows the various toxins, genins and accompanying steroid products, and references to the more recent literature. A complete chemical and pharmacological bibliography up to 1937 has been given by Gessner (198). Those bufagin formulae in Table VI possessing odd numbers of carbon atoms must be regarded as doubtful.

Bufotalin, bufotalidin, bufotalinin.—Wieland's revision of the formulae of (vulgaro)bufotoxin and bufotalin became necessary following recognition of the presence of the doubly unsaturated δ -lactone ring (ultraviolet absorption spectrum, fission with ozone) (186). Bu-

¹⁹ The nomenclature is most confusing. Bufotalin, obtained by Wieland & Weil (187) in 1913, was regarded as the anhydrogenin of (vulgaro)bufotoxin when this was discovered by Wieland & Alles (188) in 1922, but is now known (186) to be the genin itself and should receive the systematic name vulgarobufogenin or vulgarobufagin. The Japanese authors Kondo and Kotake have unfortunately sometimes used the suffix "talin" as equivalent to the suffix "genin": thus gamabufotalin (189, 190, 191, 192) is identical with the gamabufogenin (i.e., gamabufagin) of Wieland & Vocke (193), but cinobufotalin is not identical with cinobufagin (i.e., cinobufogenin) (190, 194). The term bufagin, now used to describe the genins as a class, was originally used by Abel & Macht (195) to describe the substance now termed marinobufagin, and has also been used by Kotake (196) to describe an individual substance, found later (194) to be impure cinobufagin. Again, the name bufalin given by Kotake in 1928 (189, 196) to the trihydroxy compound of which cinobufagin is the monoacetate, was used by this worker (197) in 1939 to describe a new dihydroxy compound.

TABLE VI
BUFOTOXINS AND BUFAGINS

Bufotoxin	Mean lethal dose in cats μ g. per kg.	Formula	Melting point $^{\circ}$ C.	Bufagin	Mean lethal dose in cats μ g. per kg.	Formula	Melting point $^{\circ}$ C.	Toad
(Vulgaro) Bufotoxin (186)	$C_{10}H_{90}O_{10}N_4$	205	Bufotalin = Vulgaro- bufagin (186, 199)	136 ^{a*}	$C_{23}H_{36}O_6$	148	$B. bufo bufo$ =
Vulgarobufotoxin (201)	300 ^b	$C_{28}H_{90}O_{11}N_4$	202	Bufotalidin (186)		$C_{24}H_{32}O_6$	228	$B. vulgaris$ (Europe)
Gamabufotoxin (193)	$C_{38}H_{90}O_{10}N_4$	210	Bufotalinin (186)		$C_{24}H_{30}O_6$	233	
Gamabufotoxin (202)	330 ^b	$C_{41}H_{84}O_{11}N_4$	146	Gamabufagin = Gama- bufogenin = Gama- bufotalin (186, 191, 192, 193, 199, 200)	{ ... }	$B. formosus$ (Japan)
				Cinobufagin (190, 194, 203, 204, 206, 212)	{ 100 ^b }	$C_{24}H_{34}O_5$	255	$B. bufo japonicus$ (Japan)
				Cinobufotalin (190, 194)	230 ^b	$C_{20}H_{24}O_6$	212	
Cinobufotoxin (203)	360 ^b	$C_{39}H_{88}O_{11}N_4$	200	Cinobufotalidin (207)		$C_{20}H_{26}O_7$	248	$B. gargarizans$ (China) = Chan Su or Senso
				Bufalin (189, 197)		$C_{24}H_{34}O_6$	217	
				ψ -Desacetylbufotalin (200, 220, 221, 222)		$C_{24}H_{32}O_4$	235	Amorph.
Marinobufotoxin (208, 209) ..	430 ^b	$C_{38}H_{90}O_{10}N_4$	200	Marinobufagin (195, 206, 209, 210, 211, 212)	570 ^b	$C_{24}H_{32}O_5$	218	$B. marinus$ (Central America)
Marinobufotoxin (212)	$C_{38}H_{88}O_{10}N_4$	204	Marinobufagin (213)		$C_{27}H_{30}O_6$	205	$B. paracnemis$ (Ar- gentina)
				Arenobufagin (186, 214, 215)	90 ^b	$C_{23}H_{34}O_6$	220	$B. arenarium$ (Argen- tina)
Arenobufotoxin (214, 215) ..	410 ^b	$C_{39}H_{90}O_{11}N_4$	194	Arenobufagin (211)		$C_{24}H_{32}O_6$	231	$B. regularis$ (South Africa)
Regularobufotoxin (215, 216)	480 ^b	$C_{29}H_{90}O_{11}N_4$	205	Regularobufagin (215, 216)	150 ^b	$C_{25}H_{34}O_6$	236	
Viridobufotoxin (217)	270 ^b	$C_{31}H_{90}O_{10}N_4$	198	Viridobufagin (217)	110 ^b	$C_{23}H_{34}O_5$	255	$B. viridis viridis$ (Europe)
Fowlerobufotoxin (218)	800 ^b	Amorph.	Fowlerobufagin (218)	220 ^b	$C_{23}H_{33}O_6$ †		$B. fowleri$ (North America)
Quercicobufotoxin (218)	Amorph.	Quercicobufagin (218)	100 ^b	$C_{23}H_{34}O_6$	258	$B. quercicus$ (North America)
Vallicepobufotoxin (218)	Amorph.	Vallicepobufagin (218)	200 ^b	$C_{23}H_{33}O_5$	212	$B. valliceps$ (North America)

* Letters indicate bibliographical references:

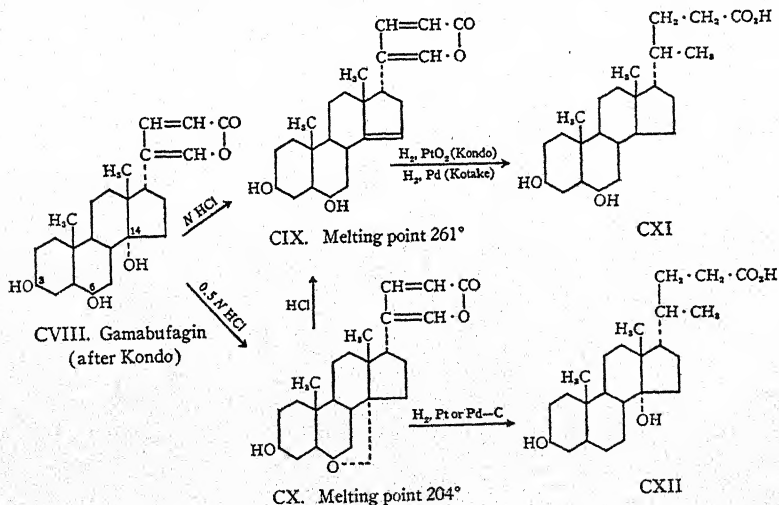
• = (236)

† Sic.

fotalidin and bufotalinin both show band maxima at 300 μ , but whereas the former affords an isocompound (as C) and so possesses a hydroxyl group at C-14, the enol-ester (as XCIX) from the latter does not undergo dehydration (186).

The "acetylgamabufalin" obtained (199) from *B. bufo japonicus* appears to be the acetylbufotalin of Wieland (188): bufotalin has also been obtained from *B. vulgaris formosus* (199).

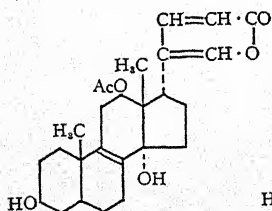
Gamabufagin.—The original formula of Wieland & Vocke (193) was modified (186) to conform with XCVIII with hydroxyl groups at 3,12, and 14, on account of the absorption spectrum; Kotake & Kubota, who placed the hydroxyl groups at 3,7, and 14, confirmed this revision by fission of the lactone ring with ozone, obtaining oxalic and glyoxylic acids and a gamabufagindiacetoxyhydroxyetiocholanolic acid (191). Wieland & Vocke (193) reported the existence of two isomeric anhydrogamabufagins—melting points 204° and 261° respectively—the former being converted to the latter by action of hot concentrated hydrochloric acid. These substances have now been further examined by Kondo & Ohno (192) who interpret their results in terms of the 3,6,14-trihydroxy formula (CVIII). Both the isomerides (CIX, CX) give absorption spectra with maxima at 300 μ , but whilst the compound CIX affords diacyl derivatives, the substance CX furnishes only a mono-*p*-nitrobenzoate. Hydrogenation of CIX and CX gives saturated lactones (as CI) accompanied by unidentified gamabufagindihydroxycholanolic acids (CXI, CXII). The



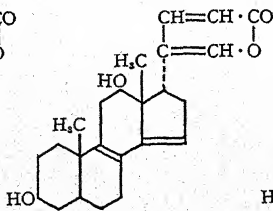
acid CXI is probably identical with that obtained from lactone CIX by Kotake & Kubota (191), who have also prepared various iso-derivatives from gamabufagin.

Gamabufagin has also been obtained by Kondo (200) from *B. vulgaris formosus*, together with the following three substances: (a) F_1 -bufotalin, $\text{C}_{26}\text{H}_{36}\text{O}_6$, (200) possibly identical with Wieland's bufotalin; (b) F_2 -bufotalin, assigned the unlikely formula $\text{C}_{25}\text{H}_{34}\text{O}_5$ (200); and (c) F_3 -bufotalin, m.p. 243° , $\text{C}_{24}\text{H}_{32}\text{O}_5$, which shows the typical absorption maximum at 300 m μ and sublimes unchanged in a high vacuum (219).

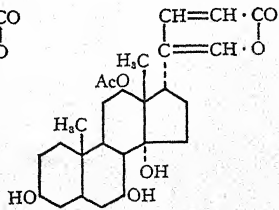
Cinobufagin, cinobufotalin, cinobufotalidin, and bufalin.—The cinobufagin, m.p. 223° , obtained by Jensen & Chen from the Chinese drug Ch'an Su (203), whose absorption spectrum, hydrogenation and precipitability with digitonin were examined by Tschesche & Offe (206), has been resolved chromatographically by Kotake (194) into two substances: (a) one, forming 75 per cent of the mixture, and for which the name cinobufagin is retained, has a m.p. 212° and the formula $\text{C}_{26}\text{H}_{34}\text{O}_6$ (compare 206, 223). Cinobufagin gives the Hammarsten reaction, affords a monoacetate, and by reduction furnishes two isomeric hexahydrocompounds, previously isolated by Tschesche & Offe (206). Ozonolysis of cinobufagin gives formic and oxalic acids as fission products of the δ -lactone ring (190, 206) and the structure CXIII suggested by Kotake is very similar to that proposed by Tschesche. With hydrochloric acid the compound CXIII gives anhydridesacetylcinobufagin (CXIV) which is hydrogenated to a saturated dihydroxylactone (as CI) accompanied by a cinobufagin-dihydroxy-cholanic acid (as CII) (204). The presence of a hydroxyl group on C-14 in cinobufagin is diagnosed by the production of isocompounds (204). (b) The second substance, cinobufotalin (CXV), m.p. 248° , was given a C_{25} -formula (194) later corrected to $\text{C}_{26}\text{H}_{36}\text{O}_7$ (190); it furnishes a diacetate, and by hydrogenation two isomeric tetrahydro-



CXIII. Cinobufagin



CXIV



CXV. Cinobufotalin

compounds (194). Cinobufotalin gives the Hammarsten reaction and the structure (CXV) is suggested (190). The absorption curves of CXIII and CXV measured separately correspond exactly with the curve given by Tschesche for their mixture.

In the preparation of cinobufagin and cinobufotalin, Kondo & Ohno (207) obtained a new substance cinobufotalidin, m.p. 217°, $C_{24}H_{34}O_6$. This is saturated and possesses no acetyl group, but exhibits the characteristic absorption maximum at 290–300 mμ. Acetylation affords a monoacetyl-monoanhydro-compound, $C_{26}H_{34}O_6$, and high vacuum sublimation gives two isomeric dianhydro-compounds distinguished by difference in volatility.

Bufalin, also isolated from Ch'an Su, was given the formula $C_{24}H_{36}O_5$ and said to give with hot acetic anhydride an acetylanhydro-compound (α - and β -forms) (189). Later (197), the formula was revised to $C_{24}H_{34}O_4$ and the compound formed by acetylation shown to be a simple monoacetate reducible to a tetrahydro-acetate. Bufalin gives a monoketone by oxidation with chromic acid, and is converted to a monoanhydrocompound by hydrochloric acid. Kotake has suggested the formula XCVIII with hydroxyl groups at positions 3 and 14.

φ -Desacetylbufotalin.—The substance φ -bufotalin, $C_{26}H_{36}O_6$, isolated by Kondo & Ikawa (220) from Ch'an Su and Chinese toads and which was regarded as the acetyl derivative of a C_{24} -compound, has been renamed φ -desacetylbufotalin, since further purification showed the substance to be $C_{24}H_{34}O_5$, to be acetyl free, and to possess the typical absorption maximum of a doubly unsaturated δ -lactone (200). The extraordinary structure proposed by Ikawa (221) therefore is no longer tenable, and the Wieland degradation on which it was based appears to be inexplicable with the new formula (as XCVIII with 3 nuclear hydroxyl groups). The new formulation is supported by ozonolysis, whereby φ -desacetylbufotalin gives formaldehyde, formic, oxalic and glyoxylic acids; the chief steroid product appears to be a ketoaldehyde, $C_{19}H_{31}O_3 \cdot CO \cdot CHO$, oxidised by peracetic acid to a ketoacid, which is converted by hydrogen peroxide to an amorphous acid, $C_{19}H_{31}O_3 \cdot CO_2H$. The methyl ester of this acid has been subjected to a Wieland degradation and furnishes a non-crystalline compound, $C_{19}H_{30}O_4$, which should be a trihydroxysteroid-17-ketone (222).

Marinobufagin.—Deulofeu (211) has shown that the formula of marinobufagin is $C_{24}H_{32}O_5$ as previously suggested by Jensen (224)

and Crowfoot (225). It gives a diacetate (211) and not a monoacetate as reported by Jensen & Evans (212). The characteristic absorption maximum at 300 m μ was observed by Tschesche & Offe (206), who also obtained evidence of a latent aldehyde group on fission of the doubly unsaturated δ -lactone ring.

PHYSIOLOGY AND PHARMACOLOGY

The references (226 to 240) cited in Tables V and VI contain many values for cardiac glycosides and toad poisons of the median systolic dose for frogs and the median emetic dose for pigeons. A few aglycones have been examined, the mean lethal doses (μ g. per kg.) for cats being: strophanthidin, 280 (230), 285 (238), 331 (232); allo-strophanthidin, indeterminable (232); digitoxigenin, 424 (238); gitoxigenin, 1850 (238); oleandrigenin, 250 (177); digoxigenin, 253 (238); in general the aglycones are less active than their glycosides, and to provide compounds of increased water solubility various amino acid esters have been prepared (241). The relation between structure and activity has recently been discussed (232). It may be noted that the bufagins are more active than the bufotoxins (240).

The cardiac glycosides have a negative influence (proportional to their concentration) on the fission of sodium β -glycerophosphate by kidney phosphatase (242), and resemble the cortical hormones in giving protection against lethal doses of potassium in cats, rats, and mice, and hindering the onset of insulin convulsions in rats and mice (243). Neither the sapogenins, cardiac glycosides, nor toad venoms possess carcinogenic activity (244).

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THE CHEMISTRY OF THE PROTEINS AND AMINO ACIDS

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This review will discuss the size and shape of protein molecules; the number and configuration of the electrically charged groups in amino acids, peptides, and proteins; and a few of the many implications of these data for chemical and biological studies. In some respects it is a continuation of an article in an earlier volume of the *Annual Review* (1). Some studies which have received brief mention in the reviews of the last three years are discussed here in more detail. A short section is devoted to the determination of serine and threonine in proteins, in which major advances have been made during the past year.

Three recent reviews in the field of protein chemistry may be mentioned, one on the specificity of proteinases and its significance for protein structure (2a); one on some general aspects of protein structure (2b); and one on the properties and functions of the plasma proteins (3).

The use of solubility as a criterion of protein purity has been greatly refined in recent years by Northrop and his collaborators. A discussion of this very important work is here regretfully omitted from lack of space. Recent studies of the purification of chymotrypsinogen (4) and of pepsin (5) may be cited; the whole field is compactly reviewed by Herriott (6). Also we shall only mention here the isolation of the sulfur-containing amino acid lanthionine (7), from wool treated with sodium carbonate, and its subsequent synthesis (8).

It is fitting here to record the great loss which protein chemistry and other fields of investigation have suffered in the death of Rudolf Schoenheimer.

AMINO ACIDS AND PEPTIDES

Electric moments and interatomic distances.—The amino acids, peptides, and proteins are members of a special class of superpolar molecules, commonly termed *Zwitterionen* or dipolar ions. Even in the isoelectric state, they bear positively charged ammonium, guanidinium, or imidazolium groups, and negatively charged carboxyl

groups. These groups are essentially ionic, and the molecules containing them possess many of the properties of ionic compounds. This is shown by their high melting points, their extreme insolubility in nonpolar solvents and their relatively high solubility in water, their high crystal densities and low apparent molal volumes in solution (1). Since they carry no net charge in the isoelectric state, their solutions are relatively nonconducting, the observed conductivity, apart from that due to the solvent, arising from the anions and cations in equilibrium with the isoelectric dipolar ion. Dipolar ions do not migrate in a uniform electric field; they do, however, become oriented in such a field, and their solutions give extraordinarily high dielectric constants (9, 10).

The most direct index of the polarity of a molecule is its dipole moment.¹ In the following discussion, such moments will be expressed in Debye units.² Evidence from several quite independent types of measurement indicates that α -amino acids have moments very close to 15 Debye units.

First we may consider the direct evidence from interatomic distances. In an α -amino acid, $^+\text{H}_3\text{N} \cdot \text{CH}(\text{R}) \cdot \text{COO}^-$, the positive charge may be regarded as located on the nitrogen atom, and the negative charge as located midway between the two oxygens of the COO^- group. From analogy with measured interatomic distances and bond angles in closely related compounds, it was possible to make fairly reliable estimates for the distance between the positive and negative charges (1). The values obtained were close to 3 Å. Recently, careful x-ray diffraction studies on glycine (12) and *dl*-alanine (13) have given a very exact picture of these amino acids as they exist in the crystal lattice. The principal features of the crystal structures are discussed elsewhere in this volume, and in this discussion we shall consider only the internal structures of the molecules. In most respects these are such as might have been anticipated from earlier studies. The two C—O distances in the carboxyl group are 1.23 and 1.25 Å

¹ The dipole moment is not always a reliable guide to the general polar or nonpolar character of a molecule. Thus *p*-dinitrobenzene has zero dipole moment, because the moments of the two highly polar nitro groups are equal and oppositely directed. Owing to the fields of force around these groups, however, it behaves in its interactions with other substances like a highly polar molecule (11). Likewise a dipolar ion such as cystine, with two positive and two negative charges, may have a dipole moment anywhere between zero and about thirty Debye units, depending on the spatial configuration of the groups.

² One Debye unit = 10^{-18} e.s.u.

in glycine, 1.25 and 1.27 Å in alanine. The probable error in these values is not more than 0.02 to 0.03 Å; hence it is possible that the two C—O distances in each amino acid are identical. In the case of alanine, however, one of the two oxygens is linked by hydrogen bonds (N—H—O bonds) to two neighboring nitrogen atoms of other alanine molecules; the other oxygen is linked by only one such bond. Hence a slight but real difference in the strengths of the two C—O bonds, and in the corresponding distances, might be expected. The O—C—O bond angle in the carboxyl group is very nearly 123° in both amino acids. The C—C distance, carboxyl carbon to α -carbon, is 1.52 Å in glycine, 1.54 in alanine; the other C—C bond in alanine is also 1.54; these are normal single bond distances. The C—N distance, however, is surprisingly low, 1.39 Å in glycine, 1.42 in alanine, as compared with a normal value of about 1.47 Å in nitromethane, dimethyl and trimethyl amines, and other compounds containing a C—N single bond. The low values in the amino acids cannot be explained in terms of any type of resonance yet known, but may conceivably be connected with the positive charge carried by the nitrogen atom. The nitrogen is uncharged in all of the compounds in which the C—N distance is 1.47. However, diketopiperazine (14) has a low N—CH₂ bond distance of 1.41 Å. Here there is a small positive charge on the nitrogen, due to the resonance present in all amide and peptide linkages. This resonance may have secondary effects on the N—CH₂ distance.

All the bond angles in glycine and alanine, except the O—C—O angle, lie between 110 and 113°. On the basis of these data, the distance between the centers of positive and negative charge is calculated as 2.95 to 3.00 Å in both glycine and alanine. This would correspond to an electric moment near 14.4 Debye units. It is, of course, an oversimplification to regard the entire charge on the molecule as being centered in the NH₃⁺ and COO⁻ groups. Undoubtedly internal polarization effects render the actual situation in the molecule more complex, but this calculated value of the moment is in excellent agreement with values deduced by other methods discussed below.

Dielectric constants and dipole moments.—The theory of Debye (15) which first provided an adequate treatment of the dielectric constants of systems containing polar molecules gave quantitatively accurate results for gases, and for dilute solutions containing a polar solute dissolved in a nonpolar solvent. Debye's treatment, however, was not directly applicable to polar liquids, in which the interaction

of molecules leads to a far more complex situation. In 1936 Wyman (9, 16) pointed out a simple empirical relation between the dielectric constants of polar liquids and the dipole moments of the molecules of which they are composed. This equation holds only as a rough approximation, and must be modified for "hydrogen-bonding" liquids like water and the alcohols. Onsager (17) then developed a theory of polar liquids which gave a satisfactory foundation for Wyman's empirical equation. Recently Onsager's analysis has been extended and generalized by Kirkwood (18) who has formulated a theory based on statistical mechanics, which should in principle be applicable to any polar liquid. In such a liquid the dipole moment (μ) of an individual molecule is in general different from its moment in the gaseous state (μ_0) because the attractions of neighboring polar molecules alter its configuration. Conversely, the neighbors of any given molecule are affected by it; their orientation with respect to its electric moment is generally not random, because electrostatic forces and steric factors prevent them from rotating freely. This tendency for the molecules to interact is expressed by Kirkwood in terms of a total electric moment, $\bar{\mu}$, which represents not only the moment of a given individual molecule, but is the vector sum of this moment plus the moments of all the neighboring molecules in the liquid. Only the first two or three shells of neighboring molecules need to be considered in evaluating this sum; more distant molecules may be regarded as distributed at random with respect to the molecule under consideration. If the immediate neighbors are also distributed at random $\bar{\mu}$ becomes equal to μ . In general, however, the two quantities may be different, both in magnitude and in direction. Kirkwood's analysis for highly polar liquids leads to the following relation between the dielectric constant, D , and the molar polarization, P , of the liquid.

$$D - 1 = \frac{9P}{2V} = 6\pi \frac{N}{V} \left(\alpha_0 + \frac{\mu\bar{\mu} \cos \vartheta}{3KT} \right) \quad (I)$$

Here V is the molal volume of the liquid, N is Avagadro's number, K the Boltzmann constant, T the absolute temperature, and ϑ is the angle between the vectors μ and $\bar{\mu}$. α_0 is the polarizability of the molecules in the liquid; it can be determined from the refractive index. For highly polar substances α_0 is small compared to the second term in the parenthesis. The high dielectric constant of water is well accounted for by equation I on the basis of the known dipole moment of water, and the orientation of the water molecules relative to one another (19).

The molar polarization of the liquid is defined in terms of the polarizability and the dipole moments of the molecules by equation I. In a solution containing several components equation I becomes

$$D - 1 = \frac{9}{2} (C_1 P_1 + C_2 P_2 + \dots) \quad (\text{II})$$

where C_1, C_2, \dots are the concentrations of components 1, 2, \dots expressed in mols per cc.

Equation II is directly applicable to amino acid solutions in which there are two components, amino acid and water. When the amino acid is dissolved in water, the polarization of the mixture is somewhat decreased by the displacement of some water molecules, but the effect of this displacement is far more than counterbalanced by the extremely high polarization due to the amino acid molecules themselves. Neglecting the decrease in polarization due to the displacement by the water, a simple relation may be obtained between the dielectric increment δ of the amino acid and the quantity $\mu\bar{\mu} \cos \vartheta$ (18).

$$\mu\bar{\mu} \cos \vartheta = 10.9 \delta \quad (\text{III})$$

Thus for glycine for which δ equals 22.6, $\mu\bar{\mu} \cos \vartheta$ is 246, μ and $\bar{\mu}$ being expressed in Debye units. If μ is taken as 15, the value derived from activity coefficient measurements on glycine, then $\bar{\mu}$ is 16.4 or only slightly greater than μ . If we define a mean moment μ_m by the relation $\mu_m = \mu\bar{\mu} \cos \vartheta$ then μ_m equals 15.7 for glycine. For glycyglycine ($\delta = 70.6$), μ_m is 27.7. Similar estimates for other amino acids and peptides give values of μ_m , which are in satisfactory agreement with the values of μ obtained by other methods.

Variation of dielectric constant with frequency (dielectric dispersion).—In an alternating field of low frequency the dielectric constant of a liquid is the same as in a static field, provided the alternation of the field is so slow that the molecules have time to orient themselves so as to follow the changing field. In an alternating field of very high frequency, however, this is no longer the case, because of the inertia of the molecules. The molecules, as a whole, cannot respond to the rapidly alternating field; only the very light electrons within them are free to respond to an alternating field of frequencies as high as those of visible light. Hence the dielectric constant at such frequencies is very low. In some intermediate range of frequency the dielectric constant D is found to vary with frequency according to the equation.

$$D = D_o - \Delta D_i v^2 / (v^2 + v_o^2) \quad (\text{IV})$$

Here $\Delta D_i = D_o - D_\infty$; D_o being the dielectric constant of the solution in a static field. D_∞ is the dielectric constant at a frequency so high that the permanent dipoles of the solute cannot follow the field at all.³ The critical frequency v_o is determined by the size and shape of the molecules, the temperature, and the viscosity of the medium. v_o for most proteins in water is found to lie in the radio frequency range, very commonly between 0.1 and 10 megacycles; for amino acids v_o is much higher, generally above 1000 megacycles (20, 21, 22). Near the critical frequency the dipole molecules are oriented by the electric field, but do not remain in phase with it. This phase difference between the field and the electric moments of the molecules is associated with absorption of energy in the liquid. This dielectric absorption can be measured by placing the liquid in a dilatometer. When the liquid is exposed to a high frequency alternating field, the heat produced by dielectric absorption causes the liquid in the capillary of the dilatometer to rise. The rate of this capillary rise, as a function of the applied voltage and the frequency, permits the calculation of the critical frequency v_o . The details of the absorption method, as applied to proteins, have been described by Shack (23).

The frequency v_o depends on the size and shape of the molecule. It is proportional to the rotary diffusion constant, Θ , which gives an index of the intensity of the rotary Brownian movement; it is inversely proportional to the relaxation time, τ .

If the molecule is a sphere of radius r , immersed in a medium of viscosity η , these quantities may be calculated from the law of Stokes for the resistance of a liquid to the rotary motion of a sphere within it, combined with Einstein's theory of Brownian movement.

$$\tau = \frac{1}{2\Theta} = \frac{4\pi\eta r^3}{kT} \quad (\text{V})$$

Ellipsoids of revolution have two different values of v_o , Θ and τ , and the dielectric constant as a function of frequency is given by a more general form of equation IV

³ It is assumed, however, that D_∞ is measured at a frequency which is sufficiently low that the solvent has the same dielectric constant as in a static field; that is, v_o (solute) $<<$ v_o (solvent), and D_∞ is measured at a frequency between these two v_o values.

$$D = D_o - \frac{\Delta D_1 v^2}{v^2 + v_{c1}^2} - \frac{\Delta D_2 v^2}{v^2 + v_{c2}^2} \quad (\text{IVa})$$

The total increase in dielectric constant produced by the solute, ΔD_t , is equal to $\Delta D_1 + \Delta D_2$. The ratio of the two critical frequencies, v_1 and v_2 , to each other, and to the value calculated for a sphere of the same volume as the ellipsoid, is a function of the shape of the ellipsoid, which has been calculated by Gans (24) and Perrin (25).

Measurements of dielectric dispersion and absorption on several amino acids in water at 65.6 megacycles (26) indicated relaxation times of the order of magnitude predicted by equation V. More recently, measurements at 1176 megacycles, in water at 23.3°, gave relaxation times (in units of 10^{-11} sec.), for glycine as 2.6, α - and β -alanine 6.6, diglycine 14.9, glycylalanine 20.3, and alanylglycine 21.3 (27). By a very different technique (28) involving a cathode ray oscillograph, at 115 megacycles, values of 12.9 for triglycine, and 43.1 for lysylglutamic acid, were obtained in water at 25°. The values for the amino acids were slightly less than those calculated, for spheres of the same volume, from V. The equation presupposes, however, that the spheres are large in comparison with the molecules of the solvent; hence it is not strictly applicable to molecules as small as amino acids, and the agreement between observed and calculated values must be considered surprisingly good. The observed values for peptides are larger than those calculated for spheres of the same volume, in agreement with their structural formulas, which indicate a somewhat elongated shape. In any case, amino acids and peptides appear to orient as rigid structures; transition from one configuration to another, by internal rotation, cannot occur in time intervals less than those corresponding to the critical frequencies (about 10^{-9} sec.) (28).

Interactions between ions and dipolar ions.—In a solution containing ions and dipolar ions, the positive end of the dipolar ion tends to attract anions around it; the negative end to attract cations. The distribution of ions around the dipolar ion is determined by the size and shape of the dipolar ion and the distribution of charge within it, by the ionic strength, by the electrostatic forces which depend upon the dielectric constant of the solution, and by the temperature (1). At very low ionic strengths, the effect of increasing ionic strength is always to decrease the activity coefficient, γ , of the dipolar ion. In dilute solution, $-\log \gamma$, for a dipolar ion, is always proportional to the

first power of the ionic strength. For a spherical dipolar ion, of radius b , with a point dipole of moment μ located at its center, Kirkwood (29) gives for $\log \gamma$ as a function of the ionic strength, in mols per liter, $\Gamma/2$:

$$B = \lim_{\frac{\Gamma}{2} \rightarrow 0} \left(\frac{-\log \gamma}{\Gamma/2} \right) = \frac{2\pi N \epsilon^2}{2303 DKT} \left(\frac{3}{2} \frac{\mu^2}{DaKT} - \frac{b^3}{a} \alpha(\varrho) \right) \quad (\text{VI})$$

Here a , the "collision diameter," is equal to b plus the mean radius of the ions in the solution, ϱ is the ratio b/a , and $\alpha(\varrho)$ is a function equal to 1.21 when $\varrho = 0.6$, and to 1.96 when $\varrho = 0.9$ [see (29), Table I]. ϵ is the electronic charge; the other terms have the same meaning as in equation I. The first term on the right of VI represents a "salting in" effect; the second represents a salting out effect, which increases very rapidly with increase in the size of the dipolar ion. The salting out term becomes relatively more important as the dielectric constant increases; it arises essentially because the dipolar ion creates a cavity, of low dielectric constant, in the solvent.

The salting out term for glycine and sodium chloride in water is calculated (29) as 0.08. An experimental value of B in VI may be obtained from electromotive force measurements (30) and freezing point measurements (31) on the system water-glycine-sodium chloride. They give, when calculated for water at 25°, $B = 0.24$. The "salting in" term in VI is thus equal to B plus 0.08, or 0.32; from which the value of the dipole moment, μ , is found to be 15 Debye units. The same value is obtained from studies on the effect of lithium chloride on the solubility of glycine in a series of alcohol water mixtures (32). The converse solubility effect—the increased solubility of slightly soluble salts in the presence of amino acids—may also be employed in the evaluation of B . Earlier studies on the solubility of thallous chloride in the presence of amino acids (33) gave values of B for glycine and alanine very close to those already given. Recently (34) the effect of glycine and alanine on the solubility of barium and calcium iodates, and of barium bromates, has been studied. The solvent effects are large; the calculated dipole distances, from Kirkwood's equations, are 4.07 Å for glycine, and 3.74 Å for alanine, both molecules being considered as spheres. The exact values obtained depend on the value of the radius assumed for the ions. The iodate and bromate ions are not spherically symmetrical, and this may be associated with the fact that the values obtained for the dipole distances are somewhat high. Silver and lead iodates in glycine and

alanine solutions give anomalously large solubilities; this appears to arise from complex formation between the metallic ion and the glycinate or alaninate ion (35).

The effect of amino acids on salt activities may also be determined by electromotive force studies on concentration cells with transference. Such studies have been reported on the cell: $\text{Ag}|\text{AgCl}|\text{KCl}(m_2)||\text{KCl}(m_1)|\text{AgCl}|\text{Ag}$, glycine (m_2) $|\text{AgCl}|\text{Ag}$. From measurements on a series of such cells, holding m_1 constant in each series and allowing m_2 to vary, the effect of the glycine on the activity coefficient of the salt is determined, leading to $B = 0.18$. This is lower than the corresponding coefficient for sodium chloride, as would be expected because of the larger radius of the potassium ion. The calculated moment of glycine was 14.4 Debye units, in very satisfactory agreement with the values obtained by other methods (36).

Kirkwood has also calculated the salting in coefficient for an ellipsoidal dipolar ion with charges $+\epsilon$ and $-\epsilon$ located at the foci, as:

$$B = \lim_{\frac{r}{2} \rightarrow 0} \left(\frac{-\log \gamma}{\Gamma/2} \right) = \frac{2\pi N \epsilon^2 g(\lambda_0) R}{2303(DKT)^2} \quad (\text{VII})$$

R is the distance between the charges, and g is a function of the eccentricity of the ellipsoid, being nearly unity if g is above 0.7. The derivation of this equation neglects salting out effects, and the finite size of the ions of the salt; but these effects appear to be of secondary importance. The salting in of ellipsoidal dipolar ions, assuming that g is constant, should thus be proportional to the first power of the electric moment. This appears to be in good accord with the facts concerning the solvent action of salts on glycine, diglycine, and triglycine in 80 per cent ethanol (37).

Another ellipsoidal model, in which a point dipole is located at one of the foci, is useful as a simple approximation to the structure of α -amino acids with a long hydrocarbon side chain, such as valine or leucine.

Electrostatic effects on dissociation constants.—The glycinium ion, $^+\text{H}_3\text{N} \cdot \text{CH}_2 \cdot \text{COOH}$ ($\text{pK} = 2.31$) and propionic acid ($\text{pK} 4.85$) are very similar in size, shape, and electronic configuration; the positive charge present in the former, however, greatly increases the tendency of the carboxyl group to dissociate. Bjerrum (38) [see also Neuberger (39)] calculated the effect of such a charge by assuming it to be immersed in a medium of the dielectric constant of the solvent, and located at a distance R from the dissociating proton of the carboxyl

group. If the charged group is in the α position, however, this calculation gives an unreasonably low value for R —about 1.3 Å in the glycinium ion. Kirkwood & Westheimer (40) have concluded that this is due to the fact that the acidic molecule itself must be regarded as forming a cavity of low dielectric constant in the solvent; this makes the electrical interactions of the groups far more intense than on Bjerrum's original hypothesis. When the calculations are made by this revised method, the distance from the $-\text{NH}_3^+$ group to the carboxyl proton in glycine is calculated as 3.3 Å, in very good agreement with the other estimates of this distance already given. Equivalent calculations may be made by comparing pK_2 of an amino acid with the pK of the ammonium group in its methyl or ethyl ester (41); the results, for a series of amino acids and peptides of different dipole moment, are in good accord with the distances deduced from dipole moments, and from the salting in effect.

Interactions between dipolar ions.—Slightly soluble dipolar ions, such as cystine, asparagine, and hemoglobin become more soluble in the presence of other dipolar ions, such as glycine. For the activity coefficient, γ_i , of a dipolar ion of species i , in the presence of another of species K , the following relation often holds (42):

$$\frac{-\log \gamma_i}{C_K} = K_R^* \left(\frac{D_0}{D} \right) - K_S^* \quad (\text{VIII})$$

Here D_0 is the dielectric constant of the solvent without component K , D the dielectric constant of the solvent containing C_K mole of K per liter. K_R^* is analogous to the salting in coefficient for the interaction between ions and dipolar ions; K_S^* , to the salting out coefficient. In general K_R^* tends to increase with increase in the dipole moments of both i and K ; it is roughly proportional to their product. K_S^* tends to increase with increase in the size of the nonpolar portions of the molecules. Equation VIII holds also when components i and K are the same; that is, when the activity coefficient of an amino acid is studied as a function of its own concentration. The very extensive studies of Smith & Smith (43, 44) on the activity coefficients of amino acids and peptides in aqueous solution furnish the most complete set of data at present available for examining the relation of structure to the interaction of dipolar ions.

Apparent molal heat capacities and electrostriction.—Strong electrolytes are distinguished by very low, often negative, apparent molal volumes and heat capacities (45, 46). This is due primarily to elec-

trostriction of the solvent; the intense electric field around an ion orients and compresses the solvent molecules in its neighborhood, so that they pack tightly around it, causing the liquid to shrink in volume. Also the normal rotational and vibrational motions of the solvent molecules are largely "frozen out" under these conditions, and this leads to a fall in heat capacity, analogous to the decrease in heat capacity when water is frozen to ice.

Electrostriction of the solvent, due to dipolar ions, is now well recognized from volume studies (1). Studies of apparent molal heat capacities, $\Phi(C_p)$, by Gucker and his colleagues, reveal the same effect even more strikingly (Table I). The value at infinite dilution, Φ_o , is

TABLE I

APPARENT MOLAL HEAT CAPACITIES OF AMINO ACIDS AND THEIR UNCHARGED ISOMERS, AS DESCRIBED BY THE EQUATION

$$\Phi(C_p) = \Phi_o + am + bm^2$$

Substance	Temperature 5°			Temperature 25°			Temperature 40°		
	Φ_o	a	b	Φ_o	a	b	Φ_o	a	b
Glycine (47)	-4.30	+9.01	-1.25	+8.83	+4.58	-0.47	+13.74	+3.27	-0.28
Glycolamide (47)	+26.55	+2.39	-0.21	+35.76	+0.62	0	+39.72	-0.52	+0.23
α -Alanine (48)	+23.75	+3.70	-0.20	+33.69	+1.48	+0.09	+36.66	+1.09	-0.22
β -Alanine* (48)	+4.04	+6.67	-0.86	+18.27	+2.64	-0.13	+23.34	+2.12	-0.10
Lactamide (48)	+52.44	-0.43	+0.05	+58.38	-0.48	+0.02	+60.92	-0.57	+0.02

* The complete equation for β -alanine (from $m = 0$ to $m = 6.37$) at 5° is

$$\Phi(C_p) = 4.04 + 6.67m - 0.86m^2 + 0.05m^3$$

All values in calories per degree per mole; m = moles solute per kg. water.

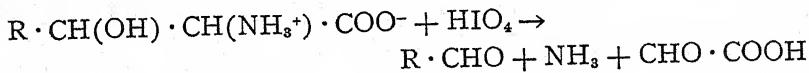
26 to 30 cal. per deg. lower for glycine, at all temperatures, than for its uncharged isomer glycolamide. α -Alanine and lactamide show the same difference. Φ for β -alanine is 20 cal. per deg. lower than for α -alanine at 5°, 13 less at 40°. The difference between the two isomers is due to the greater electrostriction produced by wider separation of the charged groups. The effect of an added CH_2 group—compare glycine and α -alanine, or glycolamide and lactamide—is to increase Φ_o by 20 to 27 cal. per deg. In homologous series of pure liquids or solids, the increment in molal heat capacity is only 6 to 8 cal. per deg. The much larger effect of the CH_2 group in dilute aqueous solutions than in pure organic liquids was pointed out several years ago (49); it is presumably associated with the breaking of hydrogen bonds holding water molecules together in liquid water, by the insertion into the

water of a hydrocarbon group which has no tendency to bond with the water.

The increment $d\Phi(C_p)/dm$ in dilute solution, denoted by " α " in Table I, is closely related to structure. It increases with dipole moment for a given set of isomers; and decreases, in a homologous series, with increase in number of CH_2 groups. A theory of apparent molal heat capacities of dipolar molecules as a function of concentration has been given by Fuoss (50) and another treatment may be derived from Kirkwood's theory of dipolar ions (51). Both theories, however, predict smaller slopes for the $\Phi-m$ curves than are given by the observed " α " values, although the general character of the observed effects is correctly predicted.

THE DETERMINATION OF SERINE AND THREONINE IN PROTEINS

Until last year, no reliable values for the serine and threonine content of proteins were known. The isolation of serine was tedious and very incomplete, and the same was true of threonine. The work of Nicolet & Shinn (52, 53) and of Martin & Synge (54), on the determination of serine and threonine by the use of periodate, appears to have yielded a simple and accurate method for determining these amino acids in protein hydrolysates. In the presence of periodate, β -hydroxy- α -amino acids react rapidly as follows:



Serine yields one molecule of formaldehyde, threonine one of acetaldehyde; and either one gives a molecule of ammonia. The two aldehydes may be separated by aeration at pH 7, the acetaldehyde passing over with the stream of gas (preferably CO_2) and being taken up in a bisulfite solution; the formaldehyde, which is not volatile under these conditions, can then be determined as the dimedon derivative. Analysis of the ammonia produced gives an independent determination of the sum of the serine and threonine present. Hydroxyproline does not give either aldehyde or ammonia with periodate. Recovery of 95 to 98 per cent of both serine and threonine in known amino acid mixtures has been obtained (52, 53). Casein is reported to contain 3.5 to 3.6 per cent threonine (53), and 5.0 per cent or more of serine (52); silk fibroin 13.6 per cent serine, and 1.5 per cent threonine (55); insulin 2.66 per cent threonine and 3.57 per cent

serine (56). As yet, the moisture and ash content of the proteins studied has not been reported, so that these figures may be subject to minor revisions.

Careful search for higher aldehydes among the periodate oxidation products from a number of different protein hydrolysates gave entirely negative results; there is thus no evidence for the existence of higher homologues of threonine in those proteins (54). Hydroxylysine, however, yields formaldehyde on oxidation with periodate, and cannot be distinguished from serine by this method of analysis, unless the two are separated by a preliminary fractionation. The "serine" values reported for gelatin thus include hydroxylysine (52, 54). Carbohydrates also react with periodic acid to give formaldehyde; hence the serine method cannot be applied directly to proteins which contain carbohydrate (52).

The ammonia yielded by periodate oxidation of casein and lactalbumin hydrolysates is equivalent to the sum of the serine and threonine, determined as aldehydes. There is thus no evidence for the presence of hydroxyglutamic acid in either protein, although casein was formerly reported to contain as much as 10.5 per cent of this amino acid (57).

THE SIZE OF PROTEIN MOLECULES

Molecular weights.—The publication of the monograph by Svedberg & Pedersen (58) completes an epoch in our knowledge of the size of large molecules. Twenty-five years ago no reliable values whatever for the molecular weights of proteins were known. Twenty years ago, when the work of Svedberg began, the only significant value available was Sørensen's estimate of 34,000 for the molecular weight of egg albumin, from osmotic pressure (59). Today the molecular weights of a large number of proteins are known with a probable error of ten per cent or less. Osmotic pressure determinations, in the hands of Sørensen, Adair, Burk, and others, have contributed greatly to this achievement, but sedimentation equilibrium, and above all sedimentation velocity and diffusion measurements, have provided most of our present knowledge. The development, in the Upsala Laboratories, of accurate methods for determining diffusion constants (60, 61), has been an essential factor in this achievement. The design, construction and operation of the oil-turbine ultracentrifuge have been described in very great detail by Svedberg & Pedersen (58); and a description of the air-driven vacuum type ultracentrifuge is given by

Bauer & Pickels (58, pp. 191-212). Recent advances in the construction of this type of instrument, particularly the electrically driven, magnetically supported, vacuum-type ultracentrifuge, have been described by Beams (62a) in a recent symposium. The optical problems of the ultracentrifuge (62b), the integration of the differential equation of the ultracentrifuge (62c), and the present status of the molecular weights of proteins (62d) were also discussed at the same meeting.

In determination of molecular weights by the osmotic pressure method, it is important that equilibrium should be attained rapidly, to decrease the danger of protein decomposition during the experiment, and to make possible a larger number of measurements in a given time. Several experimenters (63, 64, 65) have been able to reach osmotic equilibrium in a few hours to a day, depending on the size of the diffusible molecules present. Bull (65) by this method has found 45,000 for the molecular weight of egg albumin, confirming the more recent values obtained from sedimentation and diffusion, and from several earlier osmotic determinations (66).

A valuable critical survey of the methods of molecular weight determination has been given by Oncley (67). On the whole, molecular weights derived from sedimentation velocity and diffusion appear to be more trustworthy than those from sedimentation equilibrium. The attainment of equilibrium requires many days, sometimes more than a week, and the time required for its complete attainment is often difficult to determine. Moreover, alteration of the protein may occur during a long run, unless it is carried out at low temperature. Nevertheless, there are now a number of molecular weights from sedimentation equilibrium which are in very good agreement with the values obtained by the other methods.

In spite of the excellence of the techniques now employed for measuring diffusion constants, the discrepancy between different values for a given protein sometimes appears decidedly greater than would be expected from the inherent errors of the method. Thus for the undissociated hemocyanin of *Helix Pomatia*, Tiselius & Gross (60) found D_{20} , in units of 10^{-7} sq. cm. sec.⁻¹, to be 1.05, measuring concentration by the light absorption method. Polson (68) found 1.38 by a refractometric method. Brohult (69) has recently obtained 1.07 by essentially the same method, in agreement with the value of Tiselius & Gross. The difference between the two values produces a change in calculated molecular weight from 6.6×10^6 to 8.9×10^6 . All these measurements were made in the same laboratory. It is not unlikely

that different estimates for other proteins would sometimes differ as much as this.

Sedimentation constants⁴ obtained for the same protein by different observers generally agree within a few per cent. A large series of recent measurements in five different laboratories on the same preparation of centrifugally purified bushy stunt virus (70) gave a mean value of $s_{20} = 132.8$ Svedberg units, S . The lowest of eighteen values was 122.9, the highest 139.2, but most of the values lay very close to the mean. The observed variations, however, were larger than would be expected in view of the general accuracy of the technique. The virus is very homogeneous, and nearly spherical in shape, and therefore provides excellent material for critical comparisons. The diffusion constant of this virus, D_{20} , has recently been determined as 1.15×10^{-7} sq. cm. sec.⁻¹ (71). This, combined with the above s_{20} value, gives a molecular weight of 10,600,000.

Molecules which deviate greatly from the spherical shape generally show a marked variation of sedimentation constant with concentration. This is true, for instance, of tobacco mosaic virus, for which Lauffer (72) obtains s_{20} at infinite dilution as 193 S . Even more striking effects were obtained with calf thymus nucleohistone. This substance, on account of the high nucleic acid content, has the very low partial specific volume of 0.658 (73). It gives highly viscous solutions, and the molecule has evidently a very elongated shape. s_{20} , at pH 6.4, in Svedberg units, is 12.5 in 0.82 per cent solution, 24.7 at 0.1 per cent, and 31 at infinite dilution. The calculated molecular weight, from sedimentation and diffusion, and sedimentation equilibrium, is 2,150,000 (74).

Two well-defined proteins have recently been shown to have molecular weights very close to 13,000. These are ribonuclease (75) and cytochrome-*c* (76). The latter had earlier been reported (58) as having a molecular weight of 15,600, but this material included some

⁴ The sedimentation constant, s , is the sedimentation velocity in a centrifugal field of unit strength; it has the dimensions of time. A sedimentation constant of 10^{-13} sec. is denoted as one Svedberg unit, S . The measured values of the sedimentation constant are generally corrected to a standard medium, commonly taken as water at 20°, by correcting for the differences in viscosity and temperature between the medium actually employed and water at 20°. This corrected sedimentation constant is denoted by the symbol s_{20} . Similar corrections are made for diffusion constants to give the value D_{20} for diffusion in water at 20°.

impurities which could be removed by electrophoresis. The final product contained 0.43 per cent iron (one atom), and 1.48 per cent sulfur (six atoms), the molecular weight being calculated from these values.

X-ray diffraction data furnish a powerful tool for determining the molecular weights of crystalline proteins. If the size of the unit cell, and the density of the crystal, are known, the mass of the unit cell is fixed. If the cell contains n molecules, the mass of one molecule is $1/n$ times the mass of the cell. n is of course an integer, sometimes equal to unity, and always small; usually its value can be readily fixed by data from the ultracentrifuge, osmotic pressure, or chemical analysis. In general only a very rough idea of the molecular weight is needed to fix the value of n . The volume of the unit cell in a protein crystal can generally be determined within one per cent. The error in the estimated molecular weight, however, is often several times as great as this, because of uncertainties in the determination of the crystal density. Nevertheless the molecular weights so determined have furnished data which in general give an excellent independent verification of those obtained by other methods. Determinations on wet crystals of course give the molecular weight of the hydrated protein; those on dry crystals, of the anhydrous protein. Thus these studies furnish important information concerning the solvation of proteins. The available data in this field are due primarily to Bernal and his school; during the past year the work has been surveyed in two excellent reviews by Fankuchen (77) and Crowfoot (78).

Excellent x-ray diffraction data are given by ribonuclease (79), the smallest protein molecule yet to be studied by this technique. The orthorhombic unit cell contains four molecules; the molecular weight of the protein in the air-dried crystals is calculated as 15,700, which includes some water of hydration. The molecular weight of the completely anhydrous protein is estimated as 13,700, in good agreement with the value of 13,000 from sedimentation and diffusion (75). The significance of x-ray data is further considered below.

THE SHAPES OF PROTEIN MOLECULES

Knowledge of the shapes of protein molecules is far less advanced than that of their sizes. Nevertheless there is now abundant evidence that some proteins, such as hemoglobin and insulin, do not deviate very far from the spherical shape. Others, like silk fibroin, keratin,

myosin, and collagen are extremely elongated fibres.⁵ Still others, such as zein and certain antibody globulins of horse serum, are far from spherical but by no means fibrous.

The principal types of data used for estimating protein shapes are five: (i) X-ray diffraction, (ii) Frictional ratios derived from sedimentation and diffusion, (iii) Viscosity, (iv) Dielectric dispersion, (v) Double refraction of flow. The last four methods all depend upon the magnitude of the frictional resistance to the movement of a large molecule in a viscous medium. In sedimentation and diffusion the resistance to translational motion is determined; in dielectric dispersion and double refraction of flow, the resistance to rotary motion imposed by an orienting field is determined. Viscosity studies are significant because of the effect of the shape and orientation of the large molecules in altering the work required to maintain the flow of the viscous medium. A new method for studying transverse vibrations in gels, by means of the strain double refraction (81), promises to give valuable information concerning the properties of the molecules making up the gel structure.

The effect of size and shape of the molecules on these properties of their solutions is generally calculated as if they were ellipsoids of revolution. This is the simplest type of model available for purposes of calculation, but it may be quite inadequate to represent the complexity of form found in actual proteins. Nevertheless this ellipsoidal model has provided a convenient basis for calculation, which gives fairly consistent results by the different techniques mentioned above.

X-ray data and shape.—A complete description of the structure of a protein crystal would involve the specification of the exact structure of each molecule, as well as its position and orientation in the unit cell. Present knowledge does not even remotely approach this ultimate goal. Nevertheless, from the form of the unit cell, the number of molecules it contains, and the symmetry of the crystal, it is possible to impose certain restrictions on the possible shapes of the molecules.

The case of insulin is perhaps the simplest, since the rhombohedral unit cell contains only one molecule. Crowfoot (78) gives two extreme possible structures: (a) a flat rhombohedron similar to an oblate ellipsoid, height 30 Å, diameter 74 Å; (b) a prism of hexagonal

⁵ A new type of fibrous protein, different from any of these, has recently been isolated from the slime of the hagfish (80).

base 43 Å across, and height 30 Å. The true shape is probably somewhere between these extremes. Horse methemoglobin (82) [see also (78)] forms monoclinic crystals; the unit cell contains two molecules. Symmetry considerations, in conjunction with the size and shape of the unit cell, do not permit any wide derivation of the molecular shape from the spherical. Perutz (82) deduces the most probable form as a triaxial ellipsoid with $x = 22$, $y = 24$ and $z = 37.6$ Å. Setting $x = y = 23$, the axial ratio is 1.63.

The case of lactoglobulin (78) is more complicated. Both the tabular (orthorhombic) and the needle shaped (tetragonal) crystals contain eight molecules per unit cell. The evidence suggests that the molecules are not far from spherical, with an axial ratio not greater than 2:1, but a slightly greater value may be possible.

Frictional ratios and shape.—From the molecular weight and apparent specific volume of the anhydrous protein,⁶ a diffusion constant, D_0 , may be calculated for a spherical molecule of the same volume diffusing in the same medium, with no attached solvent. The ratio D_0/D , where D is the observed diffusion constant, is the frictional ratio⁷ of Svedberg, f/f_0 . This ratio is always found to be greater than unity. For ellipsoids of revolution, f/f_0 may be calculated as a function of the axial ratio of the ellipsoid a/b (84). The calculated a/b values vary rapidly for small variations in f/f_0 ; hence the calculations are not extremely accurate. The available data were first analyzed in this fashion independently by Neurath (85) and Polson (86). Their calculations were made only for elongated ellipsoids; they indicated clearly that many proteins, such as zein and *Helix* hemocyanin, are far from spherical. Their values give a maximum asymmetry factor, since the solvent attached to the molecule also lowers its diffusion constant, independently of its asymmetry. The whole situation is best represented by a graph in which values of f/f_0 are plotted as contour lines with hydration and axial ratio as abscissa and ordinate. Each f/f_0 value is compatible with a whole set of axial ratios and hydrations; if any two of these quantities are known, the third is then de-

⁶ Molecular weights determined by sedimentation and diffusion, by sedimentation equilibrium, or by osmotic pressure, are practically equal to the molecular weight of the anhydrous protein, even when the solvation of the protein in solution is quite large. See (83) and also (58, pp. 62-66).

⁷ This ratio may also be calculated from the observed sedimentation constant, relative to that of an anhydrous sphere of the same volume. The values employed for s and D should be those for infinite dilution of the protein.

terminated from the chart. The situation is fully discussed by Oncley (67).

A spherical protein, carrying 0.7 gm. water per gm. of protein in solution would have an f/f_0 value of 1.25. This is a high figure for hydration; studies of protein crystal densities indicate values of 0.2 to 0.35 gm. water per gm. protein (87). Higher f/f_0 values than 1.25 are almost certainly due at least in part to asymmetry; values below 1.1 are probably to be explained chiefly in terms of hydration. As yet no unique solution of the problem has been attained.

Viscosity and shape.—The relative viscosity, η_{rel} , of a solution containing large molecules at the volume fraction, F , depends on the shape of the solute. The ratio v :

$$v = \lim_{F \rightarrow 0} \left(\frac{\eta_{rel} - 1}{F} \right) \quad (IX)$$

gives a measure of the asymmetry of the solute. It is important that v should be measured as a limiting ratio at high dilution of solute, so that the solute molecules are free to move and rotate independently in viscous flow. For a spherical uncharged solute, Einstein showed v to be 2.5. Solutions of nonspherical molecules always give values higher than 2.5, the exact value obtained being a function of the asymmetry of the molecule. For ellipsoids, the problem is complicated by the fact that the velocity gradient in the viscosimeter tends to orient the ellipsoids parallel to the stream lines (88). If this orientation is appreciable, the viscosity falls with increasing velocity gradient; v approaches a lower limit, v_∞ , at very high velocity gradients, and an upper limit, v_0 , at zero velocity gradient (89, 90). This change of viscosity with velocity gradient is large for very elongated proteins like myosin (91) and tobacco mosaic virus (92); for most proteins, however, the degree of orientation produced by flow in any ordinary viscosimeter is so slight that all orientations may be considered equally probable, just as in a solution at rest, $v = v_0$. The most rigorous treatment of the relation of v to the shape of the solute molecule, under these conditions, is probably that of Simha (93), who gives an equation relating v to the axial ratio a/b . Values of v corresponding to various axial ratios, calculated from this equation, have been tabulated in convenient form and applied to proteins for which data are available (94).

The values of v , as calculated above, are given in terms of the volume fraction, F , of anhydrous protein. Hydration of the protein

increases the value so derived, apart from the effect of asymmetry. Here again the possible values of asymmetry and hydration compatible with a given value of v are best described by a contour line chart (67).

Careful measurements of both viscosity and diffusion have recently been made on pepsin, egg albumin, lactoglobulin, and certain serum albumin and globulin fractions; and the possible values of hydration and asymmetry were tabulated. The serum globulins were clearly more asymmetrical than any of the other molecules studied in this research (95).

Serum albumin, when dissolved in concentrated urea solutions, does not change in molecular weight, but becomes much more asymmetrical than in water, as judged from diffusion and viscosity studies (96); the results are compatible with the view that unfolding of polypeptide chains occurs in denaturation by urea (97). When the urea is removed by dialysis, the serum albumin remaining in solution is found to have reverted very nearly to the shape of the original undenatured protein (98). Urea denatured egg albumin has nearly the same molecular weight as the undenatured material, but the f/f_0 value has increased to about 4 (corresponding to an axial ratio about 90:1) indicating an extreme degree of unfolding. The molecules refold on removing the urea, but the process occurs in an apparently random way, and the system is polydisperse, with aggregates present [Rothen & Mirsky, unpublished work, cited in (62d)]. In myosin solution, on the other hand, the viscosity decreases with increasing concentrations of urea, guanidine hydrochloride, and other denaturing agents (91). This indicates that the very elongated molecules of undenatured myosin break up into shorter, less asymmetrical particles on denaturation. This is conformed by double refraction of flow measurements (see below).

Dielectric dispersion and shape.—The change of dielectric constant with frequency has already been discussed (p. 155). The same principles apply to both amino acids and proteins, and the effect was observed in proteins several years before amino acids were studied in this way. From such measurements the electric moment, and the approximate shape of the molecule, may be deduced. Since several excellent reviews are available (20, 21, 22), the subject will not be further discussed here. In Table II are given the dielectric increments of several proteins, the calculated moments, and axial ratios derived from relaxation times. Maximum values of axial ratios determined by

other methods, neglecting hydration, are also given for comparison. For a critical discussion of the shapes estimated by the various methods, see work of Oncley (67).

TABLE II

DIELECTRIC INCREMENTS, DIPOLE MOMENTS, RELAXATION TIMES, AND CALCULATED AXIAL RATIOS OF CERTAIN PROTEINS AT 25°

Protein	$\frac{\Delta D}{g}$	Mol. Wt. $\times 10^{-3}$	μ	$\tau_{H_2O} \times 10^8$	$\tau_o \times 10^8$	$\frac{a}{b}$ Disp.	$\frac{a}{b}$ S.D.	$\frac{a}{b}$ Visc.
Egg Albumin (20, 23)	0.17	44	250	18;4.7	3.7	5	3.8	5
Horse Serum Albumin (carbohydrate-free) (99)	0.24	70	380	36;7.5	6.0	6	(5.3)	5.6
Horse CO-Hemoglobin (100, 23)	0.42	67	480	8.4	(6.6)	(1.6)	5.0	4.6
Pig CO-Hemoglobin (101)	0.3	(67)	(410)	13				
Myoglobin (102)	0.15	17	170	2.87	1.45	2.6		
Horse Serum Pseudoglobulin γ (21)	1.14	142	1100	250;28	22	9	8.2	7.3
Insulin (103)	0.38	40	360	1.6			3.3	
Lactoglobulin (104, 23)	1.58	40	730	15;5.1	4.3	4	5.2	5.1
Edestin (21)	0.8	310	1400	240;27	21	9	4.4	
Gliadin (105)	0.10	42	190	27;3.8	3.1	8	10.6	
Secalin (106)	1.0	24	440	29;2.7	2.1	10		
Zein (107)	0.45	40	380	24;4.2	3.3	7	(31)	

$\Delta D/g$ = dielectric increment per gm. protein per liter; μ = dipole moment in Debye units. (1 Debye unit = 10^{-18} e.s.u.) τ_{H_2O} is the relaxation time in water at 25° (correcting for the relative viscosity of water and the solvent actually employed), τ_o = relaxation time of a sphere, of volume equal to that of the anhydrous protein, in water at 25°; $(a/b)_{Disp.}$ = ratio of major to minor axes, calculated from τ_o and observed relaxation times, by the equations of Perrin, neglecting hydration. $(a/b)_{S.D.}$ = axial ratio from sedimentation and diffusion (58); $(a/b)_{Visc.}$ = axial ratio from viscosity (94), also neglecting hydration.

Insulin was studied in 80 per cent propylene glycol; lactoglobulin in 0.5 M and 0.25 M glycine in water; edestin in 2 M glycine in water; gliadin in 56 per cent aqueous ethanol; secalin in 54 per cent ethanol; zein in 72 per cent ethanol; other proteins in water.

This table is abbreviated from a more extended one given by Oncley (22).

Double refraction of flow and shape.—The double refraction produced by flow in liquids containing highly asymmetrical particles can now be definitely related to the shape, and especially to the length, of the particles. The most important physical quantity to be measured is the angle, χ , between the stream lines and the optic axis of the flowing liquid. This angle indicates the preferred direction of orientation

of the particles. At very low velocity gradients $\chi = 45^\circ$ ($\pi/4$ radians). At very high gradients, the particles are oriented mostly parallel to the stream lines, and $\chi = 0^\circ$. When χ is near 45° , theoretical analysis (108, 109, 110, 111) leads to the limiting law for χ , expressed in radians, as a function of the velocity gradient, G , and the rotary diffusion constant, Θ , of the asymmetrical solute molecule:

$$\frac{G}{12\Theta} = \frac{\pi}{4} - \chi \text{ (if } \frac{\pi}{4} - \chi \text{ is small)} \quad (\text{X})$$

Since G and χ can be determined experimentally, Θ can be determined from equation X. For an elongated ellipsoid of revolution, of major semi-axis, a , and minor semi-axis, b , in a medium of viscosity, η , (24, 25) (compare equation V):

$$\Theta = \frac{1}{2\tau} = \frac{3KT}{16\pi\eta a^3} \left[2 \ln \frac{2a}{b} - 1 \right] \quad (\text{XI})$$

A very rough estimate of a/b , obtained by any of the methods outlined above, is sufficient to determine the factor in brackets with sufficient accuracy. For a given a/b value, Θ is inversely proportional to a^3 , and equation XI may be employed⁸ to determine $2a$, the length of the molecule.

The most direct test of these relations, on monodisperse protein preparations, is found in recent work on hemocyanin of *Helix Pomatia*. This protein, at different pH values and salt concentrations, may be obtained as whole molecules (mol. wt. 8.9×10^6), half molecules (4.3×10^6), and one eighth size molecules (1.03×10^6) (69). From the f/f_0 values, the lengths of the molecules, considered as ellipsoids, may be calculated, neglecting hydration: the values obtained are 1130, 820, and 820 Å respectively for the three sizes of hemocyanin molecule (69). The lengths calculated from double refraction of flow measurements are 890, 890, and 960 Å respectively (110). The agreement between the two sets of values is remarkably good in view of all uncertainties involved. The results indicate that splitting of the molecule takes place parallel to the long axis, so that the length is little changed in the process.

Comparable measurements on horse antibody globulin (mol. wt.

⁸ Equations applicable to oblate (disc-shaped) ellipsoids have also been derived (24, 25), but are not treated here for lack of space.

900,000; axial ratio about 20:1) give the length from sedimentation and diffusion as 960 Å; from double refraction as 1280 Å (110). The agreement is fairly good here. All these estimates of length were made from measurements of χ at angles very near 45° ($\pi/4$).

For values of χ not very close to $\pi/4$, the calculation of Θ is more complex, but, for a monodisperse solute, quite straightforward. Polydisperse systems, however, give rise to curves which are extremely various and complicated, but can be interpreted if the properties of the components are known (110, 112). Thus serum globulin, dissolved in a glycerol-water mixture, behaves in streaming, like a mixture of an optically positive protein and an optically negative lipid component. Extraction with ether and acetone at 0° apparently removes the lipid; the system then behaves like a single component. Serum globulin in water also behaves like a single component; here presumably protein and lipid are firmly united. Addition of glycerol breaks up the combination (113).

Double refraction studies on the myosins of several marine animals, together with earlier studies on mammals, leave no doubt of the great length of the myosin particles in solution, which are of the order of 10,000 to 20,000 Å for rabbit, snail, and octopus myosin (114). This is of the order of magnitude of the length of the anisotropic band in the muscle fibre. The very elongated particles of native myosin are readily broken up by many mild reagents—guanidinium salts, calcium and magnesium chlorides, lithium and ammonium salts, iodides, thiocyanates, urea—acting at low concentrations, into smaller and less asymmetrical molecules. This is shown by the almost complete disappearance of double refraction of flow in these solutions. The loss of double refraction is invariably correlated with a decrease of viscosity. The very marked changes in titratable sulfhydryl groups produced by some of these reagents (115) show no systematic relation to changes in viscosity or double refraction of flow.⁹

Sodium caseinate in 1.6 *N* Na_2SO_4 forms very large aggregates, with a length of 1600 to 2200 Å, and an axial ratio of about 5:1 as judged from viscosity and double refraction studies. In other solvents, however, the caseinate is far less aggregated (119).

⁹ The determination of sulfhydryl groups in denatured proteins, particularly egg albumin, has been studied in detail, with improved methods (116, 117); the study of 2-methylthiazoline, which yield sulfhydryl groups on hydrolysis, or on reaction with ammonium ions, promises to yield results significant for protein studies (118).

Sodium thymonucleate shows very intense double refraction of flow, and the length of the molecule has been estimated as about 4500 Å (120). The intensity of this double refraction is diminished, as in myosin, almost to the vanishing point, by the addition of certain salts, notably guanidinium halides, especially the iodide (121). The double refraction of sodium thymonucleate was almost completely restored, however, by washing out the salts, whereas in myosin the effects appeared irreversible [see, however (122)].

For tobacco mosaic virus nucleoprotein, the length as estimated by double refraction of flow (123) may be compared with the dimensions directly determined by electron microscopy (124, 125, 126, 127). The latter indicates that the fundamental virus unit is about 3000 Å long and about 150 Å in diameter. End-to-end aggregation occurs very readily, and some side-by-side aggregation is also found. The length estimated by Mehl (123) [see also (111)] from double refraction at pH 6.8 is two or three times as great as that of the fundamental unit from electron microscopy. The preparations (from Stanley's laboratory) studied by Mehl had been prepared by chemical fractionation, and were probably considerably aggregated. The preparations studied by Robinson (92) and by Wissler (120) showed even more pronounced aggregation. It would clearly be desirable to make both types of observation on the same preparation at the same time; but the data at present available indicate the approximate validity of the lengths inferred from double refraction of flow measurements.

The spontaneous parallel orientation of the tobacco mosaic virus particles, to form liquid crystals even at relatively low concentrations, is apparently explicable in terms of interionic forces. The intensive study of such systems by x-ray and optical methods (128) has already yielded results of extremely general interest, which promise to throw light on the formation of many types of oriented structure in colloidal and biochemical systems.

ACID-BASE EQUILIBRIA IN PROTEINS

Relation to analytical data.—It is possible, by suitable analysis of the titration curves of proteins, to infer the nature of the groups ionizing in different pH ranges, and thus to form an estimate of the free dicarboxylic acids, and of the histidine, arginine, and lysine content. These values may then be compared with the entirely independent determinations available from analytical data. Several different lines of evidence must be considered. (i) The approximate pK'

values of the ionizing groups may be inferred from the values for these groups in peptides of known structure (129). Thus the carboxyl groups yield their protons to base between pH 2 and 5; the imidazolium groups of histidine near pH 6 or 7; the ϵ -ammonium groups of lysine near pH 10 or 11; the guanidinium groups of arginine near pH 12 or 13; and the phenolic hydroxyl groups of tyrosine near pH 10. (ii) A maximum acid-binding capacity is attained between pH 1.5 and 2.5; this maximum should give the sum of the histidine, arginine, and lysine, along with any other basic amino acids that may be present (130). Proteins also combine with metaphosphoric acid to form an insoluble precipitate in which the phosphorus content is generally equivalent to the maximum acid combining capacity (131). A maximum base binding capacity generally cannot be accurately determined, for the guanidinium groups of arginine do not all lose their protons until a very alkaline pH value is attained. (iii) The determination of the titration curve at several different temperatures permits the calculation of the heat of ionization Q' of the groups in various pH ranges. This heat of ionization is, in cal. per mole, between +2000 and -2000 for carboxyl groups, near +6500 for imidazolium groups, and is +10,000, or greater, for ammonium and guanidinium groups. When Q' is plotted against pH, or against the mols of acid or base bound per unit of protein, the transition regions in the curve indicate the change from one type of ionizing group to another. This analysis was first employed in the case of hemoglobin (132); it has since been applied to egg albumin (133), β -lactoglobulin (134), wool protein (135), cytochrome-*c* (136), horse serum albumin (137), γ -pseudoglobulin (137), insulin (137) and casein (137). In all cases the data indicate a change from Q' values characteristic of carboxyl to those characteristic of histidine near pH 5.5, and a change from histidine to lysine values near 8.5. Thus the number of histidine groups titrated can often be fixed with considerable accuracy from the Q' values. The transition point near pH 8.5 occurs when all the imidazolium groups have lost their positive charge; the net charge on the protein under these conditions is equal to the sum of the free ammonium and guanidinium residues minus the total of the negatively charged carboxyl groups. (iv) In the presence of formaldehyde (one per cent, or thereabouts) all the ammonium groups have lost their charge when the protein is titrated to pH 8.5; the net charge on the protein is then equal to the guanidinium minus the carboxyl residues.

TABLE III
FREE DICARBOXYLIC AND DIBASIC AMINO ACIDS IN EGG ALBUMIN
AND β -LACTOGLOBULIN

Groups per mole of protein	Egg Albumin (Assumed mol. wt. 45,000)		β -Lactoglobulin (Assumed mol. wt. 40,000)	
	Titration	Analysis	Titration	Analysis
a) Total cations	41	33	46	41.5
b) [COOH]—[Ar]	37	..	52
c) [COOH]—[Ar]—[Ly] ..	15	..	18
d) [Hist.]	5	4	6	6
e) [Ly] = b—c	22	15	34	29.1
f) [Ar] = a—d—e	14	14	6	6.4
g) [COOH] = b+f	51	33	58	45-50

Data for egg albumin: titration (133), analysis (138); for β -lactoglobulin, all data from (134).

Hist. = Histidine; Ly = Lysine; Ar = Arginine.

These methods have been applied to egg albumin (133) and β -lactoglobulin (134).¹⁰ The results, set forth in Table III, show excellent agreement with the best analytical data available, for histidine and arginine, but reveal decidedly more lysine and free dicarboxylic acids than are accounted for by present analytical methods. The amino nitrogen content determined by reaction with nitrous acid agrees in both cases with the ammonium groups deduced from the titration. Also in cytochrome-*c*, which contains the extraordinarily high number of 22 lysyl residues, estimated by analysis, the amino nitrogen content is given as 32 groups per molecule (76) (mol. wt. 13,000). Sometimes, however, the amino nitrogen determination in peptides or amino acids gives falsely high values, so that some caution is required in the interpretation of these results. Vickery's thorough critical review of protein composition (138), however, indicates that the determination of lysine is far less satisfactory than that of the other basic amino acids; and also that even the best methods for dicarboxylic acids are still not adequate, especially for aspartic acid. In gelatin, the results of titrations in water, formaldehyde, and alcohol-water mixtures (139) indicate more than twice as many free dicarboxylic acids as have been

¹⁰ The name " β -lactoglobulin" denotes the globulin isolated from the albumin fraction of milk in 1934 by A. H. Palmer. The prefix serves to distinguish it from other globulins of milk; it is the same protein denoted as "lactoglobulin" earlier in this review.

isolated. The same is true of hemoglobin (138, 140). Secretin (141, 142) contains one histidyl, three lysyl, and two arginyl residues. At the isoionic point, pH 7.5, all except the histidyl must carry positive charges. Hence there must be negatively charged groups, presumably carboxyl, to balance them. However, only one asparagine and one glutamine per molecule has been reported. It may be inferred that there are other dicarboxylic acids in secretin, not yet isolated. Cannan *et al.* (134) have developed an indirect method of estimating dicarboxylic acids, based upon a differential electrometric titration of the barium salts precipitated from the protein hydrolysate by alcohol. This gives much higher results than isolation procedures, but still slightly lower values than are indicated by the titration curves.

Cytochrome-*c* contains only three histidine residues per mole (76), in striking contrast to horse hemoglobin (33 per mole). Only one of the three appears to be titrated near pH 7; the other two are apparently firmly bound to the heme iron, except in very acid or very alkaline solutions (136, 143), and have pK' values near 9.35 and 9.85, similar to that of imidazole-ferrihemoglobin (144). The nature of the heme-protein linkages in cytochrome has been very thoroughly discussed by Theorell (143). The nature of the heme-linked acid groups in hemoglobin, oxyhemoglobin, and ferrihemoglobin has been critically discussed by Coryell & Pauling (145) and by Wyman & Ingalls (146).

Combination of proteins with acid anions.—The titration curve of wool protein has been studied employing a very large number of different organic and inorganic acids (147, 148, 149). The form of the curves, from pH 1 to 6, is very similar for all acids, but the mid-point of the curve is displaced 1.5 to 2 pH units for picric and flavianic acids as compared with hydrochloric. Other acids gave intermediate curves. The data were interpreted (149) in terms of combination of the acid anions with the protein. Egg albumin gave similar effects to those of wool protein, but much less marked.

Effect of ionic strength.—As the ionic strength is varied, the titration curve of a protein tends to rotate around its isoelectric (isoionic) point, becoming steeper as the ionic strength increases. Many years ago, these effects were tentatively interpreted by Linderstrøm-Lang (150) in terms of the Debye-Hückel theory. Very detailed and careful studies of egg albumin (133) and β -lactoglobulin (134) show that a generalized form of Linderstrøm-Lang's equation works very well for these two proteins, far better indeed than could be theoretically

expected. Large sections of the titration curves can be calculated very accurately, at any ionic strength, from the general equation, the size of the protein, the number of charged groups upon it, assuming a single pK' value for each class of group.

Relation to electric mobility.—The electric mobility of egg albumin, at ionic strength 0.1, has been found to be proportional to its net charge as determined from the titration curve at the same ionic strength, over the pH range 3 to 11.7 (151). The proportionality constant, however, should from the Debye-Hückel-Henry theory be 40 per cent lower than it is found to be experimentally. A similar proportionality between mobility and charge (at ionic strength 0.02) was also found for β -lactoglobulin between pH 3 and 9 (134). Here, however, the agreement between experiment and theory was closer, the theoretical proportionality factor being 85 per cent of that observed.

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LIGNIN

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Recent reviews on the structure of lignin by von Wacek (1), Freudenberg (2), Erdtman (3), and Hibbert (4) have emphasized the essentially aromatic character of lignin and its relationship to coniferyl types.

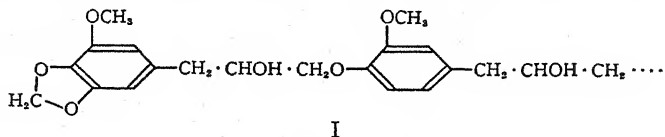
During the period 1897 (5) to 1936 (6), Klason's¹ original "coniferyl-oxyconiferyl alcohol hypothesis," in which polymers of coniferyl alcohol, coniferyl aldehyde, guaiacol aldol, etc., were included, underwent various modifications. This theory, which may be regarded as constituting the first phase of lignin research, was based on the following: the universal occurrence of coniferyl alcohol in all young plant tissue (7); the data derived from the analysis of the spruce ligninsulfonic acids and from solvent-extracted material (spruce wood) presumably containing both polymerized coniferyl and oxyconiferyl alcohol; and the occurrence of products such as catechol, guaiacol, and protocatechuic acid in lignin alkali-fusion reaction mixtures (8).

In the second phase of lignin research (1926 to 1932) emphasis was laid on new methods for the isolation of lignin from wood and on identification of functional groups (methoxyl, hydroxyl, carbonyl, aromatic nucleus) in the extracted lignins. In this connection, methods were employed involving the use of concentrated acids such as sulfuric acid (9), hydrochloric acid (10), and mixtures of hydrochloric and phosphoric acids (11), as well as much milder procedures such as alcoholysis (4) by the use of alcohols, glycols, glycerol, and glycerol chlorhydrin.

Freudenberg's earlier theory of the structure of lignin (12) was based essentially on the isolation of small amounts of catechol, protocatechuic acid, and formaldehyde from spruce hydrochloric acid lignin, and on analyses of this lignin. These results indicated the aromatic character of lignin, the absence of free phenol groups, the attachment of the methoxyl groups to aromatic nuclei, and the presence of aliphatic hydroxyl groups in side chains attached to the benzene nucleus.

¹ For review and literature survey of Klason's work see Bailey (85).

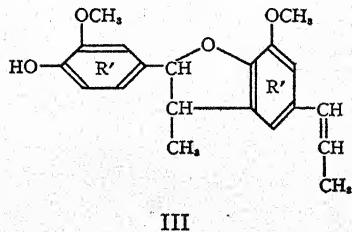
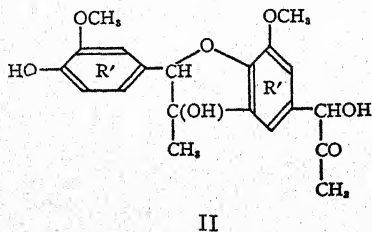
Freudenberg's modification of Klason's view of lignin as a propylphenol derivative differed only in the type of side chain envisaged and in the assumption that the building units were joined through ether linkages to give a "linear type" condensation polymer (I) (12).



Such a polymer, however, should readily undergo degradation by hydrolytic action into simple molecular units, and, this not being the case, Freudenberg found it difficult to postulate transformation into "secondary lignins" (12). The presence of the dioxymethylene group was based on the liberation of a small amount of formaldehyde, up to 1.2 per cent (13, 14, 15). The side chains visualized by Freudenberg were $R \cdot \text{CHOH} \cdot \text{CHOH} \cdot \text{CH}_2\text{OH}$, $R \cdot \text{CH}_2 \cdot \text{CHOH} \cdot \text{CHO}$, $R \cdot \text{CHOH} \cdot \text{CH}_2 \cdot \text{CHO}$, and $R \cdot \text{CHOH} \cdot \text{CO} \cdot \text{CH}_3$ (12). According to his definition these compounds are "biochemically identical" (12, p. 137); this expression, however, has, scientifically, no biochemical significance and can only be assumed to imply that they are in equilibrium with each other, at least *in vivo*.

In later investigations, in which cuproxam lignin was subjected to alkali degradation followed by methylation and permanganate oxidation, veratric, isohemipinic, and dehydrodiveratric acids were isolated in yields of 10 to 14, 2 to 4, and 3 per cent, respectively (16, 17, 18).

On the basis of these newer spruce lignin studies, Freudenberg has abandoned the ether-type linkage of assumed propylphenol units and now considers the union to be of a carbon-to-carbon type involving oxygen-ring formation between side chains and aromatic nuclei (II) (2).



(From two molecules of
 $R' \cdot \text{CHOH} \cdot \text{CO} \cdot \text{CH}_3$)

It can be seen that this structure (II) is similar to the dimer resin-type polymer (e.g., dehydrodiisoeugenol, III) suggested by Erdtman (19).

One of the chief objections to this new Freudenberg conception has been the assumed presence of the dioxymethylene group either on terminal (12) or, in his later theory, centrally disposed (2, pp. 95, 109) aromatic nuclei to the extent of 25 per cent of the spruce lignin structure. This theory has been seriously criticized (4), especially from the point of view of the nonisolation of piperonyl units. Furthermore, the inclusion of such nuclei as centrally located units in a dehydrodiisoeugenol type of polymer implies a new and unusual form of condensation. It is also of importance to note that such a condensation polymer (II) could not be formed with syringyl derivatives where the five position (*ortho* to the phenol group) is blocked by a methoxyl group.

RECENT DEVELOPMENTS IN LIGNIN CHEMISTRY

The recent developments, constituting the third phase, which have led to a clearer understanding of the structure and origin of protolignin are those connected with studies on alkaline oxidation of lignin, wood and ligninsulfonic acids, and on high-pressure hydrogenation of wood and lignin, and also with early experiments on the alcoholysis of wood. The results of these investigations have provided experimental support for the above-mentioned theoretical conception of lignin originally proposed by Klason and extended by Freudenberg, and also for the modern theory of plant respiratory catalysts as lignin progenitors developed by Hibbert (4).

ALKALINE OXIDATION OF LIGNIN AND LIGNIN DERIVATIVES

The action of alkali on ligninsulfonic acids has been investigated extensively following earlier observations (4) regarding the presence of vanillin, at least in minor quantities, in the reaction mixture. With spruce ligninsulfonic acid a yield of 6 to 7 per cent of vanillin (20) and a smaller amount of acetovanillone (21) and guaiacol (22) are obtained, while, with oak ligninsulfonic acid, in addition to these there are present syringaldehyde (23), acetosyringone (24), and 1,3-dimethoxypyrogallol (23). The addition of an oxidizing agent, such as *m*-nitrobenzenesulfonic acid, in small quantity, to the alkaline spruce ligninsulfonic acid mixture gave a lower yield of aldehyde (25). More

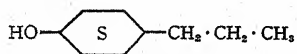
recently Freudenberg and co-workers have developed an alkali-nitrobenzene oxidation technique by which yields of 20 to 25 per cent of vanillin from spruce wood and spruce ligninsulfonic acid are obtained.

Although yields of 25 per cent vanillin appear to be the maximum obtainable from softwoods, an extension of this method to maple and aspen woods gave yields of 46 and 48 per cent, respectively, of mixtures of vanillin and syringaldehyde (27). If it is assumed that these aldehydes are derived from propylphenol units similar to those obtained by the ethanolysis of maple wood, these yields indicate that approximately 58 to 62 per cent of the protolignin in angiosperms is aromatic in nature, and thus the validity of Klason's assumption that lignin has a fundamentally aromatic structure is established.

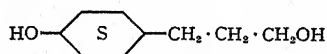
Lautsch & Piazo (28) oxidized a brominated spruce lignin with alkali and nitrobenzene and obtained 6-bromovanillin (8 per cent yield), a product which cannot be prepared directly from vanillin. This result is assumed to prove that the units in lignin are united with each other by etherification at the 4-position. Attempts to increase the yield of aromatic lignin oxidation products by substituting certain metallic oxides (29) for the nitrobenzene were unsuccessful.

HYDROGENATION OF WOOD AND LIGNIN

The preliminary work concerned with the high-pressure hydrogenation of lignin has been reviewed (3, 4). Complete liquefaction of the wood is effected by application of this technique to spruce and maple woods (30), the protolignin being converted, in part, to 4-*n*-propylcyclohexanol (IV) and 3-(4-hydroxycyclohexyl)propanol-1 (V) in yields of 19.5 and 5.8 per cent respectively (based on the Klason lignin content of the wood).



IV



V

S = Saturated

Using the carbon content of these isolated units and of the "methoxyl-free protolignin" in the wood as a basis for calculation, the combined yield of propylcyclohexane derivatives represents a recovery of 36 per cent (30).

Examination of the yields of hydrogenation products from various amorphous fractions of a maple lignin isolated by ethanolysis (31)

indicates that, for a given series of such fractions, increasing solubility and increasing susceptibility to depolymerization into simple monomolecular propylphenol units by ethanolysis are paralleled by an increasing yield of the water-insoluble propylcyclohexanol hydrogenation products. This observation is thought to indicate the prevalence of $-C-O-C-$ bonds between the propylphenol "lignin building units" in those lignin fractions which are readily soluble and are easily cleaved by ethanolysis and hydrogenolysis. Conversely, an increasing incidence of $-C-C-C-$ bonds occurs in lignin fractions having these characteristics to a lesser extent.

Hatihama *et al.* (32) hydrogenated hydrochloric acid lignin in the presence of several less active catalysts, particularly nickel, and obtained a 50 per cent yield of ether-soluble aromatic oils containing pyrocatechin and *n*-propylguaiaicol. Similar results have been reported by Freudenberg and co-workers (33) who, following earlier work by Bobrov & Kolotova (34), extended the investigations to sulfite liquor. More recently, Freudenberg & Adam (35) have developed a procedure which consists of the simultaneous dry distillation and catalytic hydrogenation of isolated lignins on the surface of which various metallic catalysts had been precipitated. In this manner ether-soluble, tarry fractions were obtained in 20 to 50 per cent yield which contained, among other products, phenols and phenolic ethers.

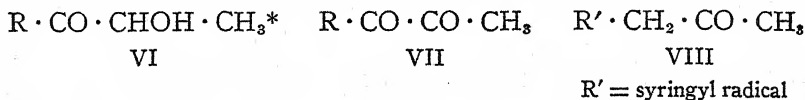
In addition to the foregoing evidence for the presence of the propylphenol unit in lignin, hydrogenation studies have shown that a relatively large proportion of the propylcyclohexane derivatives obtained from wood (30) and certain lignins (31, 36, 37) contain oxygen atoms attached to the terminal carbon atom in the propyl side chain. This result provides the only experimental proof of the presence of oxygen in that position in protolignin.

A further contribution of the hydrogenation technique to lignin chemistry has been the classification of lignins according to their relative complexities, based on the yield and nature of hydrogenation resins. The results of Adkins and co-workers (37, 38) have shown that sulfuric acid, soda, and alkali lignins are more complex than either protolignin (30) or alcoholysis lignins (31, 36).

ALCOHOLYSIS REACTION

The fourth phase in the elucidation of lignin structure may be regarded as that associated with the more recent contributions of Hibbert and co-workers on the action of ethanolic hydrogen chloride on

various plant materials (4). From the water-soluble fraction obtained by treating maple wood with 2 per cent ethanolic hydrogen chloride, and comprising approximately 35 per cent (39) of the Klason lignin content, the following products (4) have been isolated: vanillin, the α -ethoxy derivative of α -hydroxypropiovanillone (VI), vanilloyl-methyl ketone (VII), the syringyl homologs of compounds VI and VII, syringaldehyde, and presumably syringylacetone (VIII) (40).



The actual yield of these identified products corresponds to 11 to 13 per cent of the original Klason lignin content of the maple wood, and, at the present time, at least two new units, amounting to an additional 4 per cent, are in process of characterization (41). A positive relationship between these monomers and extracted lignin polymers has been established by partial degradation of ethanol lignin to the substances VI and VII by use of ethanolic hydrogen chloride (42).

As a direct result of the isolation and identification of these propyl-phenol derivatives and of 3-(4-hydroxycyclohexyl)propanol-1 (V) by means of hydrogenation, interest has been centered on the potential significance of related compounds such as β -hydroxypropiovanillone (IX), 1-guaiacyl-3-hydroxypropanone-2 (X), and their corresponding syringyl derivatives.



These derivatives have been synthesized, and their properties are being investigated (43).

Studies on the mechanism of the ethanolysis reaction and on the properties of amorphous lignin fractions obtained therefrom indicate the coexistence of polymerization and depolymerization reactions. Apparently a certain proportion of the "distillable oils" of low molecular weight produced during ethanolysis is formed by the hydrolysis of lignin aggregates of high molecular weight. Simultaneously polymerization reactions yield a complex, nonreversible, ethanol-insoluble lignin polymer. The formation of this polymer limits both the attainable yield of "distillable oils" (monomolecular units) and the degree of delignification (42, 44).

* R represents, throughout this paper, the guaiacyl and/or the syringyl nucleus.

MISCELLANEOUS EXPERIMENTS

The effect of certain variables in the Klason lignin determination has been carefully studied by Freudenberg & Ploetz (45, 46). The results indicate that this determination is, to a large extent, an arbitrary one. Of particular significance is the fact that the reaction conditions which produce the lowest yield of lignin of highest methoxyl content in the case of softwoods do not necessarily do so in the case of hardwoods. The use of hydrogen fluoride as a solvent for lignin has been suggested by Wiechert (47, 48). Preliminary investigations indicate that its use in a standardized method for the determination of lignin would eliminate many of the objections to the established sulfuric acid procedure, although the requisite expensive equipment (platinum or silver) renders its general acceptance doubtful. The difficulties encountered in applying the Klason procedure to plant products (grains, vegetables, etc.) have been emphasized recently (49), particularly with respect to (a) the apparent condensation of lignin with protein material, and (b) the higher values found when the plant material is predried at a relatively high temperature (105° C.).

Benson and co-workers (50, 51), in an attempt to establish a lignin research program of industrial significance, have commenced a study of desulfonated (with sodium hydroxide) calcium ligninsulfonate. Their recent results (52) indicate that nitrated desulfonated lignin closely resembles nitrated butanol lignin.

Butanol lignin, obtained by treating wood with butanol-water and butanol-water-alkali at 185° C., has been studied extensively by Bailey (53). He concludes (54) from comparative studies on the butanolysis of aspen and jack pine that a portion of the lignin in softwoods, but not in hardwoods, is chemically bound to cellulose. The presence of glucosidic linkages involving phenolic hydroxyl groups of the lignin is rendered doubtful by the results of investigations (55) on model substances, such as glucosides of the lignin units formed by ethanolysis.

The necessity for careful classification of lignin sources is re-emphasized by results obtained by Ritter (56) on the lignin content of various cross-sectional regions of birch trees. For example, the lignin content in a twenty-nine-year-old white birch was found to drop from 37.4 per cent at the center to 19.5 per cent at the periphery.

The similarity in the behavior of coumarin and of acetic acid lignin towards diazomethane has led Wright (57) to suggest that lignins contain a coumarin type of lactone linkage. Von Wacek & Nittner

(58) subjected beechwood tars to ozonolysis and from analyses of the reaction products concluded that substituted coumarones were present in the tars. The presence of two pyran rings in each "lignin building unit" (60) is indicated by absorption spectra studies (59) on spruce native lignin (60), spruce native lignin derivatives, lignins isolated from spruce wood by compounds containing hydroxyl and mercaptyl groups, and related compounds.

TAXONOMIC CLASSIFICATION OF BOTANICAL SPECIES

Further confirmation of the chemical differentiation between angiosperms and gymnosperms (61) has been obtained by employing the alkaline-nitrobenzene oxidation method. All investigated plant materials universally recognized as gymnosperms yielded vanillin only, while the angiosperms (monocotyledons: rye, corn, and bamboo—dicotyledons: maple, sassafras, aspen, and jute) gave both vanillin and syringaldehyde (62).

Botanically, *Ephedra*, *Gnetum*, and *Ginkgo* have hitherto been classified as gymnosperms, although the position of the first two is doubtful. Recent investigations employing the oxidation technique (63) have shown that *Ephedra*² and *Gnetum* resemble angiosperms in the chemical behaviour of their lignins, while *Ginkgo*² is definitely a gymnosperm from this point of view.

The yield of vanillin from *Ginkgo biloba* was 25 per cent, as calculated on the Klason lignin content. The yields of a mixture of vanillin and syringaldehyde, in the approximate ratio of 1 to 3, from *Ephedra trifurca* and *Gnetum indicum* were 37 and 23 per cent, respectively.

The ethanolysis method (61) and the nitrobenzene oxidation technique (62) as employed by Iwadare (64) using the wood of *Paulownia imperialis* show this plant to be angiospermic from this chemical point of view.

BIOCHEMICAL ASPECTS OF PROTOLIGNIN FORMATION

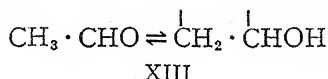
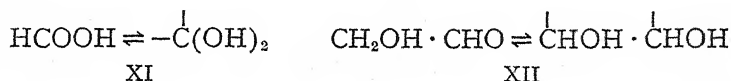
MECHANISM OF PLANT SYNTHESIS OF PROPYLPHENOL DERIVATIVES

Synthesis of simple phenols.—Various theories concerning the synthesis of phenolic compounds in plants have been based on an

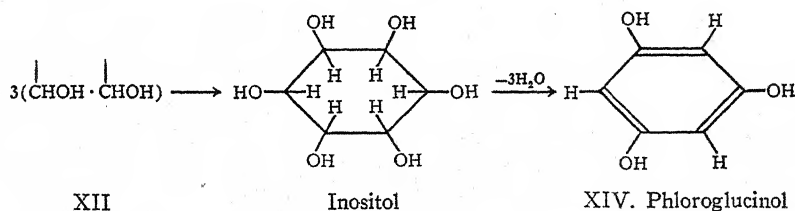
² Methoxyl values for lignins from *Ephedra procera* and *Ginkgo biloba* of 20.46 and 15.33 per cent, respectively, led Holmberg to conclude that *Ephedra* should be classified with the hardwoods (86).

assumed transformation of hexoses (65, 66). A more recent theory (4) considers the phenolic substances as being formed from intermediate photosynthetic and/or plant respiratory products.

(a) A theory based on the existence of free radicals has been proposed (67). Certain enolic equilibria are assumed to be present during the active stages of plant growth as, for example, the following enolizations of formic acid (XI), glycollic aldehyde (XII), and acetaldehyde (XIII):

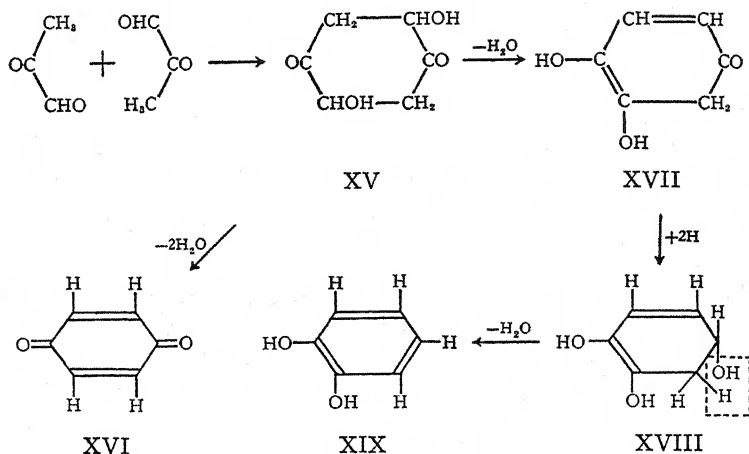


Union of the free radicals may give rise to hydroaromatic derivatives which could yield phenols by loss of water as, for example, the formation of phloroglucinol (XIV) from glycollic aldehyde (XII):

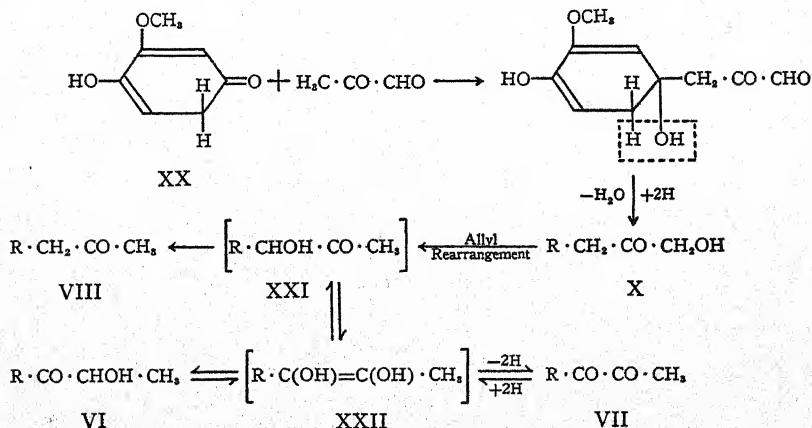


(b) A methylglyoxal theory of phenol formation has also been put forward (68). Methylglyoxal, which is a well-recognized intermediate in animal cell respiratory processes (69), presumably occupies a somewhat analogous position in plant carbohydrate metabolism (70) and has actually been isolated from a number of higher plants (71). Hibbert suggests the possibility of two molecules of methylglyoxal polymerizing to yield a cyclic dihydroxydiketone (XV) capable of undergoing the indicated reactions to give quinone (XVI) and 1,2-dihydroxy-4-ketocyclohexadiene (XVII).

This ketohexadiene (XVII) on reduction could give rise to a hydroxyenediol (XVIII) which would yield catechol (XIX) upon the loss of a molecule of water. Pyrogallol, the precursor of the syringyl nucleus, could be formed by enzymatic oxidation of catechol (XIX) just as the latter is formed from phenol (72).



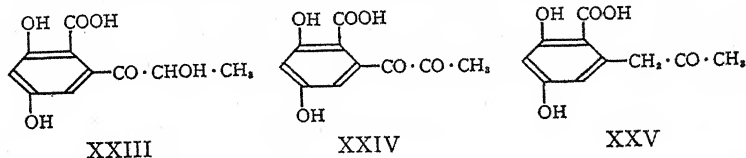
Synthesis of propylphenol derivatives.—Condensation of the “methoxylated” ketocyclohexadiene (XX) with a third molecule of methylglyoxal, followed by the loss of a molecule of water and reduction, would yield compound X. An intramolecular change involving an allyl shift in X would give the primary dismutation isomer XXI (not yet isolated) which could then yield the enediol, XXII, this in turn giving the benzoin, VI. The benzoin derivative, XXI, on reduction could yield the desoxybenzoin, VIII. Moreover the enediol, XXII, is a dihydro derivative of the 1,2-diketone, VII.



The only reaction in the series which conceivably could be open

to question³ is concerned with the rearrangement of X to XXI, but since it has been shown experimentally (43) that the veratryl derivative of X is converted into the veratryl ethyl ether of VI by ethanolic hydrogen chloride, the postulation of intermediates XXI and XXII would appear to be justified.

Experimental support for the view that these derivatives, obtained by ethanolysis, are true synthetic plant products is provided in the recent researches of Oxford & Raistrick (73) on the action of *Penicillium brevi-compactum* on glucose in acid medium, pH 4 to 5, where- by the derivatives XXIII, XXIV, and XXV are formed.



These products were not subjected to the action of chemical reagents and were isolated by simple evaporation of their aqueous solutions under reduced pressure.

The parallelism between these carboxylic acids and the products isolated by ethanolysis is remarkable. Moreover it seems probable that the acids were synthesized from phloroglucinol (1,3,5-trihydroxybenzene) by a process of plant synthesis similar to that outlined above for the formation of the corresponding guaiacyl derivatives from the keto form (XX) of 1,4-dihydroxy-3-methoxybenzene.

The introduction of the carboxyl group is not remarkable in the light of the ease with which carboxylation of phenols, e.g., resorcinol (74) and phloroglucinol (75), occurs on refluxing with aqueous bicarbonate at quite low temperatures (50–100° C.).

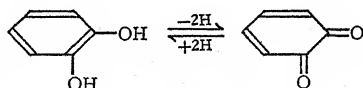
POSSIBLE SIGNIFICANCE OF LIGNIN PROGENITORS IN PLANT OXIDASE SYSTEMS⁴

Hibbert has pointed out (4) the striking similarity between the structure of the units formed by ethanolysis of lignin and the enediol plant oxidase systems of Szent-Györgyi (76). Thus α -hydroxypropiovanillone (isolated as the ethyl ether) is a benzoin and should be

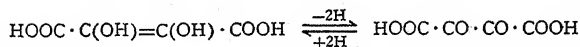
³ The products VI (as the ethyl ether), VII, and VIII have been isolated from the ethanolysis reaction mixture.

⁴ For a recent comprehensive review on plant oxidase systems see Boswell & Whiting (87).

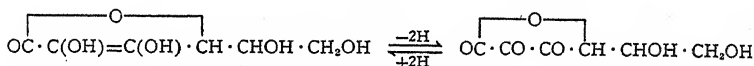
capable of undergoing the enediol dismutation changes (page 192) shown by other similar benzoin (77). These enediols may function as reductants of enediol—1,2-diketone systems, the oxidants being vanilloyl and syringoyl methyl ketones (VII) isolated as ethanoly-sis products. The similarity of such systems to those investigated by Szent-Györgyi (78, 79, 80), namely, catechol (XIX), dioxymaleic acid (XXVI), and ascorbic acid (XXVII), is apparent.



XIX



XXVI

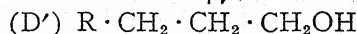
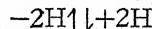
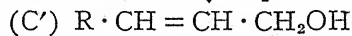
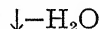
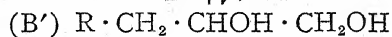
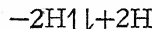
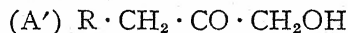
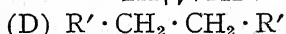
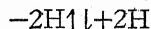
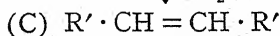
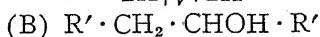
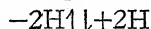


XXVII

There would also seem to be a close relationship between the C_4 aliphatic dicarboxylic acid system of animal cell respiratory catalysts (76) and a possible aromatic $C_6 \cdot C_3$ system related to lignin progenitors. In the latter system one carboxyl group is replaced by guaiacyl or syringyl, the other by the carbinol group.

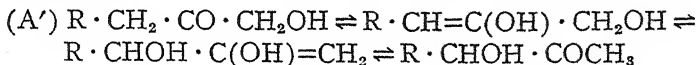
Szent-Györgyi C_4 system (animal)
($R' = -\text{COOH}$)

Hibbert $C_6 \cdot C_3$ system (plant)
($R = \text{guaiacyl or syringyl}$)

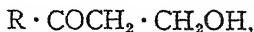


The first member of the plant system (A'), corresponding to oxalacetic acid (A), is the keto-alcohol presumably obtained as a primary condensation product from three molecules of methylglyoxal (page 192). Coniferyl alcohol (C') would be the analogue of fumaric acid (C). The passage from this $C_6 \cdot C_3$ system to the $C_6 \cdot C_3$ enediol—

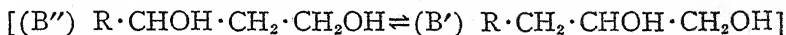
1,2-diketone system can take place by an allylic rearrangement of the oxyconiferyl- or oxysyringyl-alcohol isomer:



[see page 192 and (43)]. The isomeric form of A', namely,



may possibly function similarly to A' in the suggested $\text{C}_6 \cdot \text{C}_3$ system, while the interconvertible reduction products from these



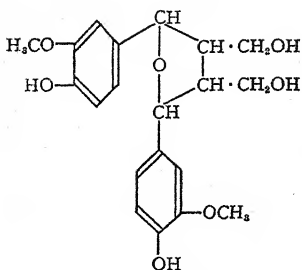
would provide analogues of citric and isocitric acids which are components of the Krebs animal cell oxidation system (81).

Experimental support for the assumption that a primary hydroxyl group is present in lignin progenitors is to be found in the experiments on hydrogenation of wood showing the presence in protolignin of large amounts of primary carbinol ($-\text{CH}_2\text{OH}$) or of methylene ether ($-\text{CH}_2 \cdot \text{O} \cdot \text{CH}_2-$) linkages.

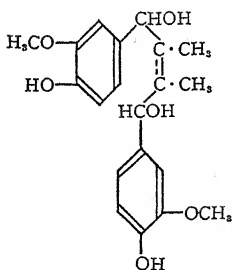
FORMATION OF PROTOLIGNIN FROM PROPYLPHENOL UNITS

Freudenberg's earlier speculations (12, p. 133) regarding the polymerization mechanism for spruce lignin building units have recently aroused greater interest in view of Erdtman's interesting researches in the field of plant resins, also the results of hydrogenation of lignin and wood at high pressures, and the isolation of $\text{C}_6 \cdot \text{C}_1$, $\text{C}_6 \cdot \text{C}_2$, and especially $\text{C}_6 \cdot \text{C}_3$ units in increased yields (4). In the absence of such experimental results Freudenberg's later speculations (2) hitherto have lacked real significance. Erdtman, in his recent review (3), points out that the structure of a wide variety of plant resins [olivil (XXVIII), lariciresinol (XXIX), conidendrin (XXX), and others] conforms to a general dimer type, the monomer being a propylphenol derivative closely related to coniferyl alcohol and the dimer formation apparently always involving the β -carbon atom.

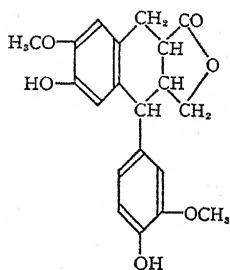
Erdtman has stressed repeatedly (3) the analogy between isoeugenol and coniferyl alcohol, and the probable existence of a close relationship between lignin and dehydrodiisoeugenol (see II and III). Haworth (82), however, believes that the absence of free phenolic groups in lignin suggests an important difference in the mechanism of



XXVIII. Olivil



XXIX. Lariciresinol

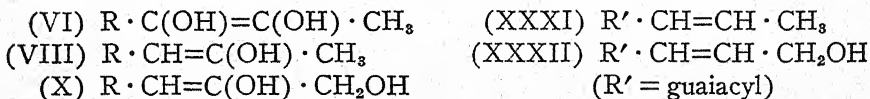
XXX. Conidendrin
(sulfite liquor lactone)

the hypothetical polymerizations of propylphenol units to plant resins and to lignin.

POSSIBLE RELATIONSHIPS OF ETHANOLYSIS PRODUCTS TO PROTOLIGNIN

Structure of lignin progenitors.—Since the ethanolysis products— α -hydroxypropiovanillone and α -hydroxypropiosyringone (VI), vanilloyl- and syringoylmethyl ketones (VII)—may only represent stabilized end products originating from dimeric types similar to dehydrodiisoeugenol, Erdtman concludes that these monomers are not necessarily the true lignin building units. That such may well be the case has been recognized by Hibbert, who has in progress investigations concerning the synthesis and properties of isomeric forms of the diketones and hydroxyketones (43).

A comparison of the side chains of enolic forms of certain propylphenol derivatives (VI, VIII, and X), now known to be directly related to the lignin complex, with the side chains of isoeugenol (XXXI) and coniferyl alcohol (XXXII) shows a close relationship:



The universal occurrence of coniferyl alcohol (XXXII) in the early period of plant growth and its absence in later periods may point to its conversion into the oxyconiferyl-alcohol type (X) during the postmortal stage.

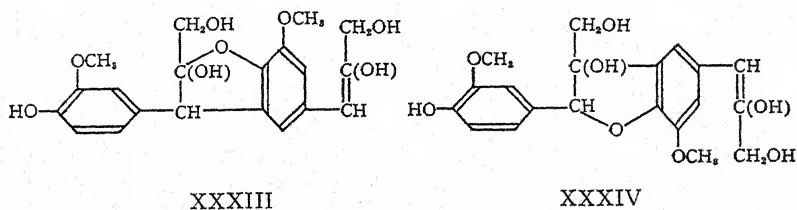
The question arises as to which of these units (VI, VIII, X, XXXII) represents the primary form or forms from which lignin is derived. The 1,2-diketones (VII) apparently do not exist as such in

the wood (40, 42); while oxyconiferyl alcohol (X), in view of its extraordinary reactivity (43) and the indicated presence of terminal $-\text{CH}_2\text{OH}$ groups in protolignin (V), is probably present along with its isomeric forms, the guaiacyl and syringyl enediol systems (see XXI, XXII, and VI on page 192).

At the moment a rational view of lignin structure would seem to indicate that protolignin is present in the plant in large measure as a series of polymers of the dehydrodiisoeugenol type (III) derived, for example, from monomeric oxyconiferyl alcohol. (Oxyconiferyl alcohol was possibly first isolated from spruce by Klason (83) and was characterized merely by empirical analysis.) During or prior to extraction by ethanolysis, these highly reactive products probably undergo partial transformation to the stabilized ethanolysis units.

Nature of polymer formation.—The tendency for the lignin progenitors (the assumed hydrogen transportation respiratory catalysts) to undergo polymerization is apparently due to the following: (a) the presence of a phenolic hydroxyl group in the *para* position with respect to a three-carbon side chain; (b) the presence, in the three-carbon side chain, of a propenyl group conjugated with an aromatic ring; (c) the pronounced reactivity of the hydrogen atom in the phenol group and of that attached to the nuclear carbon atom in the position *ortho* to the phenol hydroxyl group; (d) the reactivity of the terminal carbinol grouping; (e) the tendency of the side chain to undergo an allyl shift; and (f) the labile character of the methylene group in 1-guaiacyl-3-hydroxypropanone-2 (the keto form of oxyconiferyl alcohol) situated, as it is, between a phenyl and a carbonyl group.

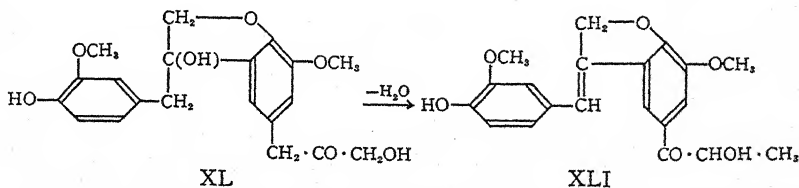
Application of the principle of dehydrodiisoeugenol polymerization (3) to oxyconiferyl alcohol (X) would yield a dimer (XXXIII or XXXIV)



which could then react further to give a trimer, tetramer, etc. Through loss of water, benzofuran polymers (XXXV and XXXVI)

It is not justifiable to assume that the dehydrodiisoeugenol type of lignin polymer is the only one capable of providing a satisfactory ex-

planation of the known experimental facts. For example, a polymer (XL) derived from two molecules of compound X would presumably undergo preferential dehydration to give XLI rather than a benzofuran derivative.



This system (XL and XLI) would account for such well-known properties of spruce lignin as (a) absence of phenolic hydroxyl groups; (b) presence of tertiary, secondary, and primary hydroxyl groups; (c) formation of labile and stable sulfonic acids; (d) formation of vanillin on oxidation; and (e) presence of terminal methyl groups. It would not explain, however, the formation of the 1,2-diketones.

It is apparent that the syringyl analogues, due to the blocking of the two positions *ortho* to the phenol group, could not function in this manner except as end-groups.

Inasmuch as coniferyl, oxyconiferyl, syringyl, and oxysyringyl alcohols may be regarded as substituted cinnamyl alcohols, and in view of the ease with which cinnamyl derivatives, in the presence of dilute acids, not only undergo the allyl shift but also form dicinnamyl ethers in high yield (84), it is possible that the syringyl units may exist as ethers in the woody tissue, thus accounting for their much readier extraction by ethanolysis and other methods.

Finally, the enediol forms of the propylphenol units under discussion (page 192) may be regarded as derivatives of styrene, and therefore possibly capable of undergoing a typical linear styrene-type of polymerization. Other types of condensation polymers, such as those involving condensation reactions between ketone and end-methyl or active methylene groups, appear less probable. The solution of this problem must evidently await much further experimentation.

In any event, the type of lignin polymerization in the case of gymnosperms appears to be much more complex than in the case of the angiosperms, the reversible type being present in the latter to a much greater extent (4). Recent work (42) has shown that both the ether-soluble and ether-insoluble ethanol maple lignins undergo degradation (depolymerization) on further treatment with ethanol-hydro-

chloric acid to give the 1,2-diketone (VII) and the keto-alcohol monomers (VI), a result in harmony with the above theoretical speculations.

It seems probable that in the case of both the lower and higher forms of plant life monomolecular propylphenols are synthesized in order that they may function as hydrogen transportation respiratory catalysts. With the lower forms these, or their more stable end products, or both, are isolable, as such, from the slightly acid medium; with the higher forms the catalysts presumably function in a similar manner but, due to the strongly oxidizing, postmortal environment, readily undergo a complex series of dehydrogenation, condensation, and polymerization reactions to yield protolignin.

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THE CHEMISTRY AND METABOLISM OF THE COMPOUNDS OF PHOSPHORUS

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Previous reviews on this subject by Kay (1), Robison (2), and Lohmann (3) have appeared in the *Annual Review of Biochemistry* for 1934, 1936, and 1938, respectively. Excellent and comprehensive reviews by Kalckar (4) and Lipmann (5) were also presented in America early in 1941. Consequently the present review will be confined mainly to the advances made in 1941, and will draw only upon previous literature that has an important bearing on the more recent developments. Special attention will be given to the chemical synthesis of compounds of phosphorus.

Analytical methods.—The King modification of the Fiske & Subbarow method for the determination of phosphorus remains the most widely used. Recently important improvements have been added to this method by Allen (6). The procedure described by Allen reintroduces the use of amidol (2,4-diaminophenol hydrochloride) (7, 8, 9) as the reducing agent. Certain precautions are described which largely overcome the difficulties previously encountered in the use of this reagent.

Among the newer developments for the analytical determination of phosphorus, improved methods have been described by Stern (10) based on polarographic procedures, and by Fontaine (11) based on the use of the spectrophotometer.

Methods of synthesis.—In a period when the importance of the phosphoric acid esters was becoming more and more apparent, it was desirable to improve the old method of Emil Fischer, namely, the reaction with phosphorus oxychloride in the presence of pyridine and similar reagents used to neutralize hydrochloric acid. Brigl & Müller (12) used successfully diphenylphosphoryl chloride ($\text{C}_6\text{H}_5\text{O}$)₂POCl, in the presence of pyridine, especially for the phosphorylation of carbohydrates and partly blocked carbohydrates, for instance, 2,3,4,5-diacetone fructose.

The phenyl groups of the diphenylphosphoric esters thus formed are split catalytically with platinum oxide and hydrogen. Independently of these authors, Brederick, Berger & Ehrenberg (13) used the same method for the synthesis of nucleotides. Diphenylphosphoryl chloride has also been used by Zeile & Meyer (14) in attempts to synthesize creatinephosphoric acid.

A similar method has been used by Zervas (15) for the synthesis of tetraacetylglucose-1-phosphate. Tetraacetylbromoglucose was treated with the silver salt of dibenzylphosphoric acid, and the protecting benzyl groups were removed by catalytic hydrogenation. The product thus obtained by Zervas was subsequently shown to be a derivative of the α,β -isomer of the Cori ester, by the work of Wolfrom & Pletcher (20, 22), which is discussed later in this review under the Cori ester.

Lynen (16) synthesized acetylphosphate by treating acetyl chloride with the silver salt of dibenzylphosphoric acid and subsequent catalytic removal of the benzyl groups.

Mention may also be made here of a method by Zetzsche & Büttiker (17), which uses derivatives of mono- and dianilidophosphoric acid for phosphorylation, but the authors themselves admit that their expectations of its success have so far been only partially fulfilled.

An interesting phosphoric acid derivative of formaldehyde, $\text{CH}_3 \cdot \text{COO} \cdot \text{CH}_2 \cdot \text{OPO}_3 \text{Ca} + \text{H}_2\text{O}$, was synthesized by Pratesi (18) by the action of sodium phosphate on chloromethylacetate.

Cori ester (glucose-1-phosphate).—Of all the biologically important sugar phosphates occurring in nature, the Cori ester is perhaps of prime importance because of its role in the initial reactions of carbohydrate metabolism. Further important work has been carried out in the past year with regard to its nature. The chemical synthesis of this ester, originally reported by Cori, Colowick & Cori (19), who obtained the amorphous barium salt, has been repeated by Wolfrom & Pletcher (20) and the ester isolated as the crystalline dipotassium salt. [Kiessling (21) had previously isolated the same dipotassium salt from natural sources.] A convincing proof of structure was obtained by Wolfrom & Pletcher from the chemical behaviour of the dipotassium salt, and the structure of the ester previously assigned by Cori *et al.* (19) was confirmed.

Wolfrom, Smith, Pletcher & Brown (22) have recently described the synthesis of the α,β -isomer of the Cori ester, by the use of the

phosphorylation technique of Zervas (15). By comparison of the rotation of this isomer with that of the Cori ester, it is now evident that the natural Cori ester is the α -form of *d*-glucopyranose-1-phosphate. Wolfrom and co-workers (22) point out that this constitutes an example of an α -form occurring in nature and the corresponding β -form occurring only synthetically, in contrast to the usual experience with glycosides. They also indicate the possible relationship of the synthetic β -form to cellulose, analogous to the relationship of the α -form to starch and glycogen.

The role of the Cori ester in carbohydrate metabolism, including the reversible enzymic synthesis of starch (23) and glycogen (24) has been described in a recent extensive review by Cori & Cori (25) and by C. F. Cori (26).

An outstanding success has been obtained by Green, Cori & Cori (27) in the isolation in crystalline form (as an adenylic acid complex) of the muscle phosphorylase which catalyzes the reversible reaction: polysaccharide + $\text{H}_3\text{PO}_4 \rightleftharpoons$ glucose-1-phosphate. The enzyme is present to the large extent of about 2 per cent of the total of the protein of the extract. The corresponding starch-producing potato phosphorylase of Hanes (23, 28) has been obtained in a highly concentrated form by Green & Stumpf (29) by a series of successive ammonium sulfate fractionations.

Regarding the mechanism of glucose-1-phosphate synthesis from glucose and from fructose, Sutherland, Colowick & Cori (30) have demonstrated that the purified enzyme from muscle extract, phosphoglycomutase, is capable of converting glucose-6-phosphate to glucose-1-phosphate, which latter compound can then be converted to glycogen by the action of phosphorylase.

It has been demonstrated by Bear & Cori (31) that the polysaccharide synthesized by the action of muscle phosphorylase on glucose-1-phosphate gives an x-ray diffraction pattern very similar in structure to that shown by natural plant starches, while the polysaccharides synthesized by heart and liver phosphorylase yield diffuse patterns, closely resembling glycogen in this respect. These observations substantiate previous evidence based on solubility properties and iodine coloration (32). Astbury, Bell & Hanes (33) had previously shown that native potato starch granules and polysaccharide granules prepared by the action of potato phosphorylase on glucose-1-phosphate give the same x-ray diffraction pattern.

An important difference between natural starch and synthetic

starch prepared from Cori ester by the Hanes potato phosphorylase has been reported by Hassid & McCready (34). Unlike the natural starches, the methylated synthetic starch yields, on hydrolysis, no detectable end-group (tetramethylglucose) or dimethylglucose. This indicates a difference in molecular constitution between the synthetic and natural polysaccharides.

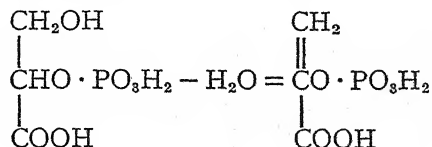
An activator in the hexokinase system has been obtained by Colowick & Kalckar (35) in the form of a heat- and acid-stable protein present in muscle tissue. It is apparently a promoter for the reaction $\text{adenosinediphosphate} + \text{hexose} \rightarrow \text{adenylic acid} + \text{hexose-6-phosphate}$.

With regard to the source of energy required for the phosphorylation of glucose, Colowick, Kalckar & Cori (36) have demonstrated that, with cell-free heart muscle and kidney extracts, this energy may derive from the oxidation of glucose itself to carbon dioxide and water. For every mole of glucose which is completely oxidized, six additional moles of glucose disappear. With heart muscle extract, five of these six additional moles were identified as fructose diphosphate. In kidney extracts, aerobic glycolysis occurs and a large part of the hexose diphosphate formed from glucose is converted to lactic acid. The mechanism of the oxidation of glucose in these extracts is traced in relation to the Krebs citrate cycle (37), and to the phosphorylation and oxidation mechanisms proposed by Negelein & Brömel (38), Warburg & Christian (39), Lipmann (40), and Ochoa (41).

Acetylphosphate.—The important work of Lipmann (5, 40) regarding the role played by acetylphosphate in bacterial oxidation has shown further progress. Lipmann (42) has succeeded in isolating acetylphosphate (as the mercury salt) from the oxidation products of pyruvic acid in the presence of inorganic phosphate by the action of the lactic acid bacteria, *Bacterium Delbrückii*.

Phosphopyruvate.—An extensive study of the relationship between phosphorylation and oxidation of pyruvate in pigeon-brain preparations has been reported by Ochoa (43). It was observed that not more than half of the esterification of inorganic phosphorus with either hexose monophosphate or glucose occurring in brain dispersions when pyruvate is oxidized (with fumarate as catalyst) can be related to the oxidation of a dicarboxylic acid, and the rest of the esterification is connected with the oxidation of pyruvate itself. The connection between the oxidation of pyruvate and the phosphorylation of adenylic acid and creatine (44) is discussed.

Enolase.—In continuation of earlier work, namely, of the isolation of the reducing fermentation ferment (45) and the oxidizing fermentation ferment (39, 46), Warburg & Christian (47)¹ have now isolated and crystallized a third ferment of yeast fermentation, enolase, which was discovered by Lohmann & Meyerhof (49). The function of that ferment in fermentation is the transformation of 2-phosphoglyceric acid, by elimination of water, into 2-phosphopyruvic acid.



It was shown that enolase is not a simple protein, but a protein complex formed by a combination of protein and metallic salt. Thus, the protein when freed from metallic salt is catalytically inactive. It becomes a ferment only on addition of salts of zinc, manganese, or magnesium. In the case of a ferment preparation where it is not known which of the three metals is combined with protein, it is possible to find this out by inhibition tests. Zinc enolase, but not manganese or magnesium enolase, is inhibited by hydrocyanic acid. Magnesium enolase, under otherwise equal conditions, is much more sensitive to fluoride than manganese enolase. The enolase which is active *in vivo* is probably magnesium enolase, as can be concluded from such inhibition experiments.

Since the authors were unable to crystallize the free protein of the ferment or its magnesium compound, they have found that the mercury salt of the enolase crystallizes easily and nicely.

The mercury salt is catalytically inactive, even on addition of magnesium salts, by which the mercury cannot directly be replaced. But the elimination of the mercury can be carried out completely and without damage to the protein by dialysis against hydrocyanic acid. In this way there is obtained the pure free ferment protein, which, after saturation with magnesium salt, converts, per molecule and minute (pH 7.4): at 20°, 10,000 molecules substrate; at 38°, 30,000 molecules substrate.

A study of the chemical mechanism of the fluoride inhibition of

¹ Because of the importance of the Warburg-Christian papers and the present difficulty of obtaining European publications, the work is described in some detail.

fermentation has also been reported by Warburg & Christian (48). The specific reversible inhibition of fermentation by fluoride consists, according to Lohmann & Meyerhof (49), in the inhibition of the ferment enolase by fluoride, for, of the fluoride-sensitive fermentation ferments, enolase is the most sensitive. The chemical mechanism of this inhibition has up to now been obscure. Warburg & Christian made quantitative investigations of fluoride inhibition by means of the optical enolase test (absorption of the ultraviolet wavelength 240 μ . by enolic 2-phosphopyruvic acid). It was shown that fluoride inhibition was determined by three substances: fluoride, magnesium salt, and phosphate. If the magnesium concentration was kept constant, and the fluoride and phosphate varied, the authors found the formula:

$$c_{\text{phosphate}} \times c_{\text{fluoride}}^2 \times \frac{\text{residual action}}{\text{inhibited action}} = K_{(\text{constant})} \quad (\text{I})$$

If the phosphate concentration was kept constant and the fluoride and magnesium salt varied, the authors found the formula:

$$c_{\text{magnesium salt}} \times c_{\text{fluoride}}^2 \times \frac{\text{residual action}}{\text{inhibited action}} = k_{(\text{constant})} \quad (\text{II})$$

For the range of concentration investigated, the following conclusions were drawn: (a) The substance responsible for fluoride inhibition is a complex magnesium-fluoro-phosphate. (b) The magnesium-fluoro-phosphate inhibits the action of the ferment by combining itself, proportionally to its concentration, with the ferment protein, the resulting complex being capable of dissociation.

For the equations (I) and (II) can also be written:

$$\frac{c_{\text{magnesium-fluoro-phosphate}} \times c_{\text{free protein}}}{c_{\text{complex}}} = k_1$$

Since the free magnesium salt originates the enolase action by combining itself with the ferment protein, and since the magnesium-fluoro-phosphate stops the enolase action by combining itself with the ferment protein, evidently the specific reversible fluoride inhibition consists in the removal from the protein of the active magnesium salt by the complex magnesium-fluoro-phosphate.

Also, zinc enolase and manganese enolase are inhibited by fluoride, under otherwise equal conditions, but only by considerably higher

concentrations of fluoride than are required to inhibit magnesium enolase. These inhibitions have not as yet been investigated thoroughly.

Crystalline fermentation ferments from tumors.—The fermentation ferment from Jensen sarcomas from rats which catalyzes the last stage of tumor fermentation, namely the reduction of pyruvic acid to lactic acid, has also been isolated as the crystalline mercury salt by Kubowitz & Ott (50) in Warburg's laboratory. As with the mercury salt of enolase, the mercury can be removed by dialysis against hydrocyanic acid. The pure active ferment thus obtained converts 80,000 molecules of pyruvic acid to lactic acid per molecule per minute at 38°. For the purpose of comparison, the authors prepared the crystalline "normal" ferment, which is responsible for the pyruvic acid to lactic acid reduction in the working muscle from the muscles of rats of the same breed. They point out that a comparison of these two ferments with the same chemical function, one of pathological origin and one of normal origin, should decide the important question regarding their chemical similarity or dissimilarity.

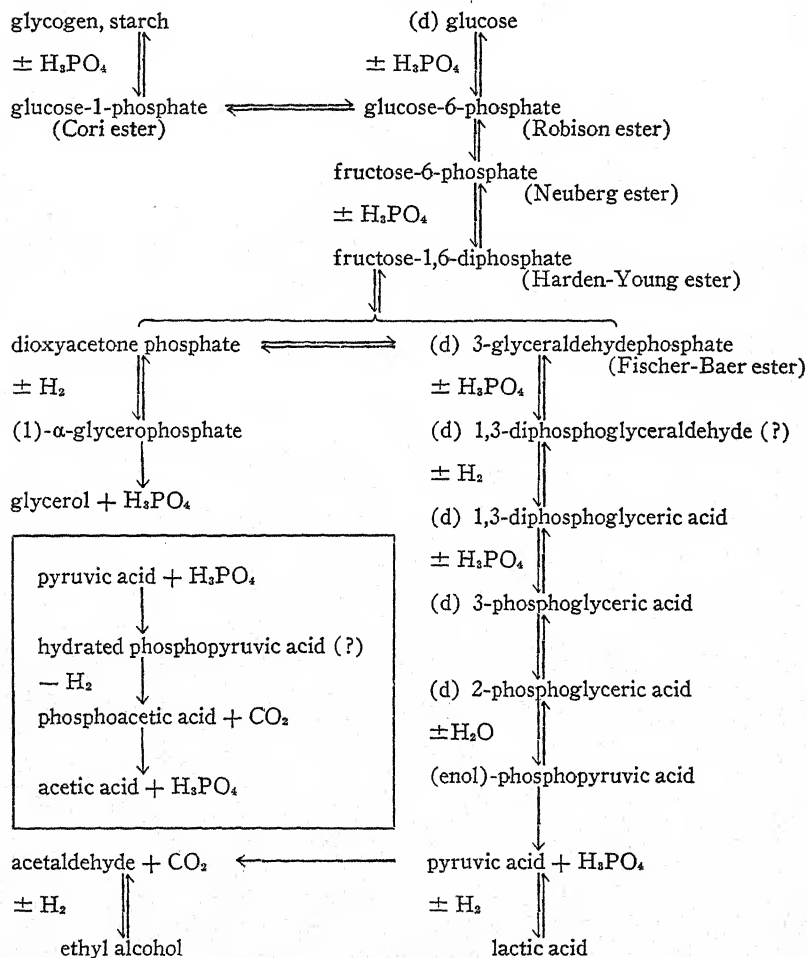
Fermentation scheme (Embden-Meyerhof scheme).—The well-known work of Meyerhof and his collaborators on alcoholic fermentation and glycolysis has been extensively dealt with in the reviews of Lipmann (5) and Kalckar (4). In recent lectures in this country, Meyerhof (51) has traced the chronological development of our knowledge of the fermentation and glycolysis process and outlined the basis for the most recent additions to and expansions of the Embden-Meyerhof scheme. Since the scheme was last stated in this *Review* (Robison, 1936) (2), several important incorporations have been made.

The self-explanatory diagram on page 210 illustrates the present status of the scheme, as depicted by Meyerhof (51). The inserted portion outlines the oxidative decarboxylation of pyruvic acid to acetic acid and carbon dioxide (40), in which the unstable intermediate, acetylphosphate, plays a role analogous to that of 1,3-diphosphoglyceric acid in the main scheme.

The significance of oxidations for muscular contraction has been reviewed by Meyerhof (52) with a discussion of how far the older statements can be upheld today.

In a paper on the inhibitory effect of glyceraldehyde on glycolysis Stickland (53) confirms that in muscle extract glycolysis of starch is not greatly inhibited by high concentrations of glyceraldehyde, but on the other hand he finds that glycolysis by this system in the pres-

SCHEME I



ence of hexokinase is completely inhibited by 0.003 *M.* glyceraldehyde. This inhibition is reversed by a small excess of hexokinase and small amounts of pyruvic acid.

Stickland also points out that according to Lehmann & Needham (54) the dimeric form of glyceraldehyde inhibits glycolysis of glycogen in muscle extract at the stage, $\text{glycogen} \rightleftharpoons \text{Cori ester}$, while the monomeric glyceraldehyde has no such effect.

O'Kane & Umbreit (55) demonstrated that during glucose fer-

mentation by resting cells of *Streptococcus faecalis*, the changes in phosphorus distribution seem to be in accordance with the theories of phosphorylating glycolysis.

Carboxylase.—Green, Herbert & Subrahmanyam (56) describe the purification and properties of carboxylase. The enzyme was isolated from top brewer's yeast as a diphosphothiamin-magnesium-protein, and contained, at the highest purity level reached, 0.46 per cent diphosphothiamin and 0.13 per cent magnesium. Kubowitz & Lüttgens (57) also studied the composition, cleavage and resynthesis of carboxylase. They found, for their most active enzyme preparation, 7 gram atoms of magnesium and 1 gram molecule of thiamin for every 75,000 grams of protein.

The preparation of a carboxylase from pea roots has been described by Horowitz & Heegaard (58).

Cozymase.—Jandorf has described a simplified method for the isolation of pure diphosphopyridine nucleotide (cozymase) (59).

Jandorf, Klemperer & Hastings (60) have outlined a manometric method for the determination of diphosphopyridine nucleotide which is based on the catalysis by cozymase of the breakdown of hexose diphosphate in the presence of arsenate and the purified aqueous muscle extract.

A synthesis of cozymase by *Chilomonas paramecium* is described by Hutchins, Jandorf & Hastings (61).

Cocarboxylase.—In a series of experiments on the oxidation and reduction of thiamin and diphosphothiamin [the latter prepared by the method of Weijlard & Tauber (62)] Guzman Barron & Lyman (63) found that the chemical passivity of thiamin toward oxidation-reduction is considerably strengthened by phosphorylation, while its activity as an enzyme component is manifested only after phosphorylation.

In *in vitro* experiments with avitaminotic animals, Guzman Barron, Lyman, Lipton & Goldinger (64) observed that thiamin, probably as diphosphothiamin, accelerates condensation reactions of pyruvate leading to the synthesis of carbohydrate, of α -keto-glutarate, of citrate, of acetoacetate, and of succinate. The role of the thiamin in these processes is discussed. Moreover, it was observed (65) that the oxidation and utilization of α -keto-glutarate by the tissue of avitaminotic rats were increased by the addition of thiamin which had previously been subjected to phosphorylating conditions.

Acetyl methylcarbinol system.—An important new enzyme system

from *Aerobacter aerogenes* has been described by Silverman & Werkman (66). The enzyme, which acts only at an acid pH, is responsible for the synthesis of acetylmethylcarbinol from pyruvic acid. In contrast to carboxylase the enzyme, which does not use acetaldehyde, requires inorganic phosphorus, and the formation of a phosphorylated intermediate is indicated. The system represents an isolated pyruvate mechanism and differs from any previously reported.

Cytochrome-c reductase.—Haas, Horecker & Hogness (67) have reported the isolation from top-yeast of a new flavoprotein, cytochrome-*c* reductase, which completes the oxidation-reduction chain between hexose monophosphate and cytochrome-*c*. The prosthetic group is alloxazine mononucleotide. The enzyme is remarkably sensitive to acetone and dioxane as well as to low pH and heat.

Adenosinephosphates.—Kerr (68) describes the isolation of adenosinetriphosphoric acid as the dibarium salt, from dog brain, with a yield of 70 per cent. Szent-Györgyi & Banga (69) confirm the results of Engelhardt & Ljubimowa (70) that adenosinetriphosphate is a part of myosin. Ljubimowa & Pevzner (71) have described the smooth and almost quantitative conversion of adenosinetriphosphoric acid to adenosinediphosphoric acid by the action of reprecipitated myosin. In further studies on the relationship of myosin to the adenylic acid system, Needham, Shen, Needham & Lawrence (72) have reported on the effect of adenylypyrophosphate upon the flow birefringence of myosin. A new method for the determination of small amounts of adenosine-5-phosphoric acid and its homologues, based on the coenzyme properties of these compounds in the enzymatic splitting of phosphopyruvic acid, has been described by Schlenk & Schlenk (73).

α -Glycerophosphates.—The action of phosphatases of various origins on the enantiomorphic α -glycerophosphates has been studied by Baer & Fischer (74). The *d*(+)- α -glycerophosphoric acid prepared from *l*(-)-acetone glycerol was hydrolyzed with a markedly greater velocity than the biologically occurring *l*(-)- α -glycerophosphoric acid.

Radioactive phosphorus and phosphorus turnover.—Radioactive phosphorus has been employed to study the effect of phlorhizin on the phosphorus turnover in rat tissues. Rapoport, Nelson, Guest & Mirsky (75) found that the turnover of the pyrophosphate fraction in the kidneys of phlorhizinized rats showed a marked decrease. Independent work by Weissberger (76) however showed no difference in the

phosphorus turnover of phlorhizinized and normal rats, in the kidney, intestine, blood and liver, or in the renal, intestinal or hepatic phospholipid. No separation of inorganic phosphorus and organic (acid-soluble) phosphorus was performed.

Fries and Chaikoff (77) also used radioactive phosphorus to study the effect of size of dosage and age and size of animal on the uptake of phosphorus in the rat. With animals of the same size, the uptake of P^{32} by blood and brain remained constant, irrespective of the volume of solution or amount of phosphorus injected. The metabolism and distribution of phosphorus in the brain was also studied.

Phytic acid.—Inositolhexaphosphoric acid (phytic acid) has been found by Rapoport (78) to occur in large amounts in chicken blood and also in turtle blood. It accounts for approximately half of the organic acid-soluble phosphorus in the erythrocytes of the chicken.

In extending these studies, Rapoport & Guest (79) examined the concentrations of organic acid-soluble phosphorus compounds in the blood cells of forty-six vertebrate species. With the exception of the turtle, phytic acid was found in considerable amounts only in avian species.

Phosphopeptones.—A phosphopeptone containing 3 moles of glutamic acid, 3 moles of isoleucine and 4 moles of serine has been isolated in the form of its barium salt, by Damodaran & Ramachandran (80) by digestion of "paranuclein" from casein with trypsin.

Lowndes, Macara & Plimmer (81) obtained a caseophosphopeptone from "Rhenania" casein. They ascribed to it, according to its analysis, the composition of an octapeptide containing 2 phosphoryl groups, 2 moles of glutamic acid, 2 moles of serine, 1 mole of a dicarboxylic acid, and 3 moles of an amino acid which is probably isoleucine. The peptone differed from that of Damodaran & Ramachandran (80).

Rimington (82) published a note on the amino acid present in phosphopeptone isolated from casein by tryptic digestion. His results indicate a nonapeptide and in a later preparation a decapeptide. Both peptides contain, according to their analytical data, 5 moles of glutamic acid, 4 moles of serine, and 3 moles of phosphoric acid.

A paper by Plimmer (83) deals with the preparation of phosphotyrosine, phosphohydroxyproline, phosphothreonine, phosphoserine, and phosphoisoserine by phosphorylation of the amino acids with H_3PO_4 and P_2O_5 . All esters are hydrolyzed by 1 N HCl at 100° , and by phosphatases of kidney and intestine.

Owing to the ubiquitous occurrence of phosphoric acid compounds in nature, the scope of the present review makes no pretence to be all-inclusive. An attempt has been made to include only the recent work not fully incorporated in previous reviews of this subject, nor yet belonging to the field covered by other reviews in this volume.

Nature appears to use phosphorus as an activator for reactions between organic compounds, especially carbohydrates, and enzymes, probably since the phosphorus residue is able to become readily attached to the protein of the enzyme. An additional and extremely important function of phosphorus is indicated by the recent discovery that certain anhydrides between phosphoric acid and organic acids, e.g., acetic and glyceric acids, play important roles in biological reactions, suggesting to the organic chemist that nature uses certain reagents which are in some sense comparable to the classical chemical reagents used *in vitro*, such as the organic acid halides and organic acid anhydrides.

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CARBOHYDRATE METABOLISM

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PHOSPHORYLATION

Phosphorylation of sugars, a process of basic importance in all phases of carbohydrate utilization, in absorption and in the anabolic and catabolic phases of metabolism, was the subject of further studies, mainly by Cori and his collaborators. A missing link in glycogen synthesis was found by the discovery that the conversion of glucose-1-phosphate to glucose-6-phosphate is a reversible process. The enzyme which catalyzes the reaction, phosphoglucomutase, was isolated from phosphorylase and isomerase, the other two enzymes known to be involved in the formation and breakdown of glycogen (1). Green & Stumpf (2) have purified potato phosphorylase and found it to differ from animal phosphorylase in not requiring adenylic acid for its action and in not being inhibited by phlorhizin. The polysaccharide it forms *in vitro* lacks the catalytic properties of natural starch. Bear & Cori (3) characterized in detail the polysaccharide formed by muscle phosphorylase *in vitro*. It closely resembles potato starch in distinction from the product formed *in vivo* by organisms of the same species that served as the source of the muscle phosphorylase. The cause of this anomaly is unexplained.

Carbohydrate phosphorylation and oxidation in cell-free dialyzed kidney extracts were described by Colowick *et al.* last year. Now they report (4) that in kidney extracts and in heart muscle the complete oxidation of every mole of glucose to carbon dioxide and water is accompanied by the disappearance of six additional moles, five of which can be accounted for as fructose diphosphate. This indicates that at least ten of the hydrogen atoms involved in the complete oxidation of glucose are linked with phosphorylation. In kidney extracts aerobic glycolysis occurs as a secondary reaction; a large part of the hexose diphosphate which disappears is recovered as lactic acid. In kidney extracts the oxidation of succinate leads to the phosphorylation of mannose and adenosine with conversion of the former to fructose diphosphate and of the latter to a mixture of adenylic acid and adenosine polyphosphate. Anaerobic transfer of labile phosphate groups in the presence of monoiodoacetate from adenosine triphosphate to glucose

takes place in heart muscle extract but has not been demonstrated in kidney extracts.

A heat-stable protein, isolated from muscle tissue, has been found to activate hexokinase to bring about the reaction: adenosine diphosphate + hexose \rightarrow adenylic acid + hexose-6-phosphate (5, 6). One μ g. of the purified protein effects the transfer of at least 14 μ g. of labile nucleotide phosphorus in 5 min. at 30°. It is precipitated by trichloroacetic acid, sodium sulphate, and ammonium sulphate. Boiling in 0.1 N HCl for 20 min. does not alter its activity; alkalis or peroxide inactivate it, but the activity is restored by glutathione or cysteine; pepsin destroys it. The relative yields from various tissues are as follows: muscle, 100; heart and brain, 5; kidney, liver, and baker's yeast, zero.

Brain dispersions, in the presence of catalytic amounts of adenylic acid and the remaining components of the pyruvate oxidation system, catalyze the conversion of hexose monophosphate or glucose to hexose diphosphate by the anaerobic transfer of phosphate from phosphopyruvic or phosphogluconic acids (7). Ochoa infers that the oxidation of pyruvate is linked with the phosphorylation of adenylic acid to adenosine triphosphate which then transfers its labile phosphate to glucose or hexose monophosphate. He found that when pyruvate oxidation is inhibited by arsenite, the ratio of phosphorus esterified to oxygen utilized is one half of that prevailing with pyruvate-fumarate in the absence of arsenite. From this he concluded that the catalytic action of C₄-dicarboxylic acid alone accounts for only half of the phosphorylation connected with pyruvate oxidation. According to Geiger (1940),¹ highly diluted cell-free brain extracts form lactic acid from glucose or fructose but very little from hexose phosphates, which indicates that the esters are not normal intermediaries of glycolysis in brain. Ochoa (8) found that hexose monophosphate and diphosphate are as readily glycolyzed as glucose. Although hexose diphosphate is glycolyzed without the addition of phosphate acceptor, addition of creatine increases the rate of lactic acid formation. This enzymatic process occurs in cell-free preparations of muscle, retina, and other tissues as well as of brain. Hexokinase activity, i.e., the transfer of phosphate from adenosine triphosphate to glucose, was more clearly demonstrable in acetone-dried powder of brain.

¹ Almost all papers published prior to 1941, to which reference is made in the text, have been excluded from the bibliography. In such cases the year of publication is given in the text.

Macfarlane & Weil-Malherbe (9) assume that glycolysis in brain proceeds by a cycle of phosphate transfers. This is based on the observation that in rabbit brain slices during aerobic glycolysis inorganic phosphate remained unchanged while the pyrophosphate decreased to one third of its original level.

DIGESTION, ABSORPTION

Beazell (10), studying the rate of saccharification of starch in the stomach, found that gastric digestion of starch is by far more significant than it is generally thought to be. Quantitatively it seems to be more extensive than the gastric digestion of proteins. In the rat, absorption of glucose from the stomach is inhibited by alcohol (11). The attendant pylorospasm could not be ascribed to irritation of the gastric mucosa, since the alcohol was administered intraperitoneally; the phenomenon therefore is attributed to central nervous system irritation.

MacKay & Clark (12) report that the rate of intestinal glucose absorption in the rat may, under certain conditions, greatly exceed the figures previously known. Thus, rats rapidly consuming large amounts of glucose, without forced feeding, after a period of fasting, showed absorption coefficients as high as 300 (milligrams of glucose per 100 sq. cm. of body surface). Similar results were obtained when the appetite of the rats was stimulated by injections of protamine zinc insulin. By increasing their appetite through exposure to a cold environment, the rats consumed large amounts of food rich in glucose with the consequence that the absorption coefficient rose to over 500. Sinclair & Fassina (13) found that rats suffering from a deficiency of essential unsaturated fatty acid, this condition being produced by raising them on low-fat high-carbohydrate diet, absorbed glucose at a rate 50 per cent higher than that of normally fed control animals. In mature rats, on the other hand, fed for three weeks or longer on a high-fat diet, a pronounced decrease in the rate of glucose absorption was observed. The rectal absorption of glucose in man is only one gram per hour, and is probably the result of simple diffusion, due to the absence of the phosphorylating mechanism (14).

The rates of absorption of glucose, fructose, galactose, and xylose in the toad are not affected by hypophyseal or adrenal insufficiency, in apparent conflict with Verzár's theory (15). Addition of thiamin, riboflavin, nicotinic acid and pyridoxin has no effect on the activity of the gastrointestinal tract of dogs already on an adequate diet, but

dried yeast or its 50 per cent alcohol extract increases the rate of digestion and absorption by 20 per cent. This effect, at least partly, is ascribed to pantothenic acid (16).

GLYCOGENESIS

After it had been found that carbon dioxide could serve as building material in synthetic reactions in animal tissues, Conant *et al.* (17) demonstrated that such processes occur in the normal mammalian organism. They fed rats lactic acid in which the carboxyl carbon was radioactive (C^{14}). Formation of hepatic glycogen accounted for 32 per cent of the lactate; only 1.6 per cent of the radioactive carbon, however, was present in the liver glycogen. This fact indicates that the principal pathway of glycogen formation did not lead via unbroken three-carbon chains. Proceeding on this basis, Solomon *et al.* (18) gave to fasted rats unmodified lactate orally, followed by the intravenous injection of radioactive bicarbonate. The glycogen deposited in the liver contained from 0.3 to 1.1 per cent of the radioactive carbon, derived from carbon dioxide (bicarbonate), while none of it was present in muscle glycogen. On the basis of work by Krebs & Eggleston, the authors suggest possible reactions involving the formation of oxalacetic acid and phosphopyruvic acid as the pathway over which carbon dioxide enters in the conversion of lactic acid to glycogen.

Gemmell has demonstrated (1940) *in vitro* that insulin enhances glycogen formation in muscle tissue. His further studies with the same experimental technique (19, 20) have yielded additional information. It was found that only part of the glucose that is taken up by the tissue from the fluid medium is recovered as glycogen; a considerable portion is unaccounted for. When glucose was added to the medium, up to 200 mg. per cent, the respiratory quotient rose, but further increase in glucose concentration was without effect. The oxygen consumption of the tissue was the same in the absence as in the presence of glucose; this indicates that glucose is preferentially oxidized, taking the place of some other metabolites. Insulin increased the glucose uptake by the tissue, as regards both glycogen formed and the fraction unaccounted for. The effect of insulin was evident at higher (500 mg. per cent) as well as at lower (200 mg. per cent) glucose concentrations. The respiratory quotient and oxygen consumption, however, were not affected by insulin, a fact which led Gemmell to the conclusion that "the main function of insulin is to aid

deposition of glycogen in the muscle from glucose in the medium." This action of insulin was found to be specific for glucose. Galactose, fructose, maltose, succinate, lactate, citrate, xylose, and arabinose did not serve as substitutes.

Hechter *et al.* (21), employing Gemmill's technique, obtained partially contradictory results. Working with glucose concentrations that ranged from 100 to 400 mg. per cent, they found that while at low concentrations insulin enhanced glycogen formation considerably, at 400 mg. per cent glycogen was formed at virtually the same rate in the absence as in the presence of insulin. On this basis it was concluded that "insulin cannot be regarded as a *specific* accelerator of the storage or the oxidation of carbohydrate." Glycogen formation from glucose *in vitro* has been shown to occur also in liver slices (22). The rate of the process increases with increasing concentrations of glucose in the medium. The optimal concentration, 3 to 4 per cent, is much higher than in the living organism.

Excessive hyperglycemia after glucose feeding of "diabetic" rats (Yale strain) is linked with a diminished ability of the liver to store glycogen. Rats showing normal glucose tolerance deposited considerably more glycogen in the liver than did the Yale strain, but there was no difference between the two groups as to the glycogen storing ability of the muscle tissues (23). The hyperglycemia induced in rats by feeding of ammonium chloride (lowering of blood pH) occurs at the expense of the glycogen stores of the liver, the amount of muscle glycogen remaining unaffected (24). Both of these findings emphasize the primary role of the liver in the regulation of the glycemie level.

Conversion of substances other than hexoses to glycogen, as for example citric acid, is well known. Keuther & Smith (25) report the quantitative recovery of fed citrate as liver glycogen in the albino rat. The rate of intestinal absorption of citric acid is proportional to its concentration. MacKay *et al.* (26) found racemic alanine to be as good a source of glycogen for the liver of the rat as the *l*(+)-isomer, when the two were fed in identical quantities. Previously (1940) they reported these isomers as being equally efficacious as antiketogenic factors. The *d*(-)-isomer, when fed alone, formed glycogen at a much lower rate than the same amount of the *l*(+)-form, and thus much of the former was excreted in the urine unutilized. In apparent conflict with this finding Butts *et al.* (27) found for tyrosine that the *l*(-)-isomer was a glycogen former in rats, whereas the racemic form,

according to a previous report by these authors (1938), yielded no significant glycogen deposits. Sorbitol fed to monkeys (28) increased liver glycogen, in distinction from mannitol. In line with this is the finding that two hours after the ingestion of crystalline sorbitol the respiratory quotient in man was increased to the same level as it was by glucose feeding.

As regards the effect of hormones and related substances, Corey & Britton (29) report that glycogenolysis in the isolated rat liver is more effectively inhibited by perfusion with adrenocortical extract plus glucose than with glucose alone. Perfusion of cat liver with glucose-Ringer-gum solution containing adrenocortical extract effected an increase of from 50 to 100 per cent in the glycogen content, occasionally in only ten or fifteen minutes. According to Koepf *et al.* (30) adrenocortical extract increases glycogen synthesis in rat liver slices from pyruvate and from *D*-lactate (but not from alanine and glutamic acid). In intact rats, fasted for twenty-four hours, the glycogen content of the liver was significantly increased within an hour after the intraperitoneal injection of adrenocortical extract. Liver slices from adrenalectomized rats (22) formed from glucose only about one fifth as much glycogen as those from normal rats. Pertinent observations were made by Russell & Wilhelmi (31) to the effect that kidney slices from adrenalectomized rats formed less carbohydrate substance from *DL*-alanine, *L*(+)-glutamic acid, and α -ketoglutaric acid than those from intact rats. Depression of the rate of deamination is suggested as the inhibitory factor.

The effect of epinephrine on glycogen formation in the liver was studied by Bendall & Lehmann (22). The two-phase action of epinephrine was reproduced in liver slices, i.e., under conditions where lactic acid from the muscles could be ruled out as a possible source of the increase in liver glycogen during the second phase of epinephrine action. When liver slices were first incubated for sixty minutes with 10^{-5} *M* epinephrine, without glucose, then for thirty minutes with one per cent glucose, only the second phase was manifest, as exemplified by an increase of 45 per cent in liver glycogen.

Several pituitary preparations described by Neufeld & Collip (32) produced an increase in the glycogen content of the tissues of fasting animals, mainly of the liver, provided that the injections were given preceding a fast. The effect was most marked in the mouse, less so in the rat, guinea pig, and rabbit. Subcutaneous implants of diethyl stilbesterol, estriol, or estradiol (33) enhanced hepatic glycogen for-

mation in rats. The muscle glycogen was not affected. At the same time the size of the liver and the insulin content of the pancreas also increased.

INTERMEDIARY REACTIONS, OXIDATION

The formation of α -ketoglutaric acid from pyruvic acid with the participation of carbon dioxide has been recognized by several workers, but the mechanism of the reaction is a controversial subject. Krebs & Eggleston (34) believe that pyruvate condenses with carbon dioxide forming oxaloacetate which, reacting with another molecule of pyruvate, forms citrate, and the reaction then goes on to α -ketoglutarate. Evans (35), on the basis of experiments with labeled carbon dioxide ($C^{18}O_2$), believes that citric acid is not an intermediate in the synthesis. Wood *et al.* (36) also reject the citrate pathway. The heavy carbon participating in the reaction was present exclusively in the carboxyl in alpha position to the ketonic oxygen, a finding that was corroborated by Evans & Slotin (37).

Wood & Werkman have shown as early as 1935 that carbon dioxide participates in synthetic reactions in all forms of life. Using heavy carbon dioxide in experiments with microorganisms, their observations led to the conclusion that succinic acid was formed from pyruvic acid and carbon dioxide, and propionic acid by decarboxylation of a symmetrical dicarboxylic acid (38, 39), and that labeled carbon was present solely in the carboxyl group of the acids (39, 40).

In brain suspensions washed with phosphate buffer until the residual oxygen uptake was 10 per cent or less of the original, added glucose, pyruvate, or lactate increased the rate of oxidation (41); 2,3,5-triiodobenzoate completely inhibited the no-substrate oxidation and cut in half the rate of oxidation of the substrates named. It had very little effect on the oxygen uptake of liver and kidney. The concentrations at which one half of the maximum rates of oxidation obtain for glucose, mannose, maltose, and fructose, increased in the order given; washed rat brain suspension was used. Iodoacetate, 2,3,5-triiodobenzoate, and fluoride inhibited the oxidation of all four sugars. Glucose and mannose inhibited the oxidation of maltose and fructose (42).

Decarboxylation of pyruvic and α -ketoglutaric acids is catalyzed by a crude enzyme preparation obtainable according to Green *et al.* (43) from the heart, brain, or kidney of pig and from horse heart and pigeon breast muscle. Carbon dioxide is evolved both aerobically and

anaerobically. An enzyme preparation which catalyzes the breakdown of oxaloacetate to pyruvate and carbon dioxide was extracted from *Micrococcus lysodeikticus* (44). Magnesium ion is the activator necessary for the reaction. Still (45) found that cell-free extracts of *Escherichia coli*, containing no carboxylase, oxidize pyruvate to acetate and carbon dioxide. The dehydrogenase system comprises a protein (or proteins), inorganic phosphate, cocarboxylase, and magnesium or manganese. Barron & Friedemann (46) describe great differences in the oxidation systems of various bacteria.

The effect of thiamin on the pyruvic acid content of blood and urine has been the subject of a number of studies in laboratory animals and man. The results all indicate that carbohydrate feeding in thiamin deficiency increases the pyruvic acid concentration in blood as well as in urine, permitting the inference that pyruvic acid is an obligatory intermediate in the catabolism of glucose under normal physiological conditions. It was shown in 1939 that thiamin deficiency in rats leads to an immediate increase in the bisulfite-binding power of the urine and that administration of thiamin corrects the anomaly, the decrease of bisulfite-binding matter being proportional to the amount of thiamin administered (47). Harper & Deuel (48) found a progressive increase of pyruvate in rat urine with the progress of thiamin deficiency, and a drop in pyruvate excretion after optimal thiamin administration. Amounts of thiamin adequate for minimal growth failed to abolish completely the abnormal excretion of pyruvate. Shils *et al.* (49) confirmed the existence of a close relationship between pyruvate excretion and the state of alimentation. Feeding of carbohydrates or other glycogen-forming substances, as lactic acid (Banerji), to thiamin-deficient rats invariably increases pyruvate excretion. On carbohydrate-free diets there is a decrease but no return to normal. In thiamin-deficient dogs (50) pyruvate is increased as in rats; intravenously injected glucose produces in such dogs higher and more protracted hyperglycemic levels than in normal animals.

Bueding *et al.* (51) found moderate but distinct increases in the blood pyruvate content of healthy, normally nourished men, within one half to one hour after ingestion of glucose. Thiamin-deficient individuals show in the postabsorptive state blood pyruvate levels that are above the normal range of 0.77 to 1.16 mg. per cent; after glucose feeding their blood pyruvate increases to markedly higher levels and the elevated level is sustained longer than in normal individuals (52).

ENDOCRINE FACTORS

Insulin.—Experiments carried out with tissue slices, which were discussed in a preceding chapter, show that although insulin is not indispensable for glycogen formation, it does increase the rate of the process in tissues. The rate of carbohydrate oxidation, however, does not seem to be affected by insulin (20, 21, 22). The reaction $\text{glucose} \rightleftharpoons \text{glycogen}$ can be reproduced in cell-free enzyme systems without the intervention of insulin. On this basis Cori (95) advances the hypothesis "that these hormones are not essential components of enzyme systems and that their effect of accelerating or inhibiting certain enzymatic reactions is dependent on a more or less intact cell structure."

A new, extremely sensitive technique for the assay of insulin was elaborated by Gellhorn and his associates (53). They report that intraperitoneal injection of 0.02 unit per 100 gm. of body weight causes convulsions or coma in the adrenodemedullated rat, 0.025 to 0.03 unit in the hypophysectomized rat, but as little as 0.001 unit has the same effect on rats after combination of the two operations. Addition of blood does not interfere with the insulin effect. With this method the insulin content of normal human blood, two and one half hours after a meal, was estimated as 0.02 unit per 100 cc., of normal dog blood, and 0.01 unit per 100 cc. after a twenty-four-hour fast. Blood drawn after pancreatectomy of a dog appeared to be free of insulin (54).

Mirsky *et al.* (55) report that the carbohydrate metabolism of the duck, as gauged by the customary methods, shows no deviation from the normal after pancreatectomy. The animal suffers a loss of weight which can be checked by the feeding of raw pancreas or pancreatin. On prolonged fasting the duck develops hunger diabetes which, as manifested in the glucose tolerance curve, does not differ from that of the normal duck. This indicates that lack of insulin plays no part in the decrease of glucose tolerance during starvation. Starvation ketosis, and ketonemia after phlorhizin administration, are paradoxically greater in the normal than in the depancreatized duck.

Himwich and his associates (56) continued their studies on brain metabolism during insulin hypoglycemia. The changes in blood flow in the absence of convulsions are not significant; the arteriovenous oxygen difference, however, indicates that the metabolic rate of the brain may drop to one fourth of the normal level. Injection of glucose

increased the arteriovenous oxygen difference; lactate and pyruvate were less effective.

Best & Haist (57) observed that the injection of insulin diminishes the insulin content of the rat pancreas. It had been shown previously that either fasting or fat feeding decreases the pancreatic insulin; it is now demonstrated that the superimposition of insulin injections further accentuates the phenomenon in both conditions.

Pituitary.—Detailed studies on the effect of injections of anterior pituitary extracts in dogs (58) showed considerable individual differences as regards the time and the amount of extract required for the development of permanent diabetes. Some dogs responded positively after the injection of relatively small doses of extract over periods of from nine to fourteen weeks, while others failed to respond after such periods even to large doses. Partial pancreatectomy, not enough to produce diabetes, caused the refractory animals to respond with permanent diabetes to the injection of small doses of anterior pituitary extract. In the permanently diabetic dogs, fasting or fat feeding temporarily diminished the symptoms of diabetes. The finding is confirmed again that the diabetes of these dogs is due to a loss of function of the islands of Langerhans. Another study of Young dogs (59) revealed that protein (meat) feeding exerted a far greater ketogenic effect than fat feeding. During meat feeding the urinary excretion of nitrogen was greater than the nitrogen intake and ketonuria much greater than during either fasting or fat feeding. Meat feeding markedly increased the oxygen consumption without affecting the respiratory quotient. The basal oxygen consumption in the Young dogs was significantly increased above the prediabetic level.

Houssay *et al.* (60) compared the effect upon the toad of anterior pituitary extracts from a number of species. The diabetogenic action of the preparations decreased in the following order: man, dog, toad, white rat, guinea pig, hen, steer, snake, fish. The hereditary diabetes of a certain strain of rats (Harned) suggested a histologic study of the anterior lobe of the pituitary (61). No significant difference from the normal was found.

According to Griffiths (62) posterior lobe extract prevents the hypoglycemic action of subcutaneously injected insulin in rabbits, but this antagonistic action is absent if the insulin is administered intravenously. This suggests that the antagonistic effect is due to a decreased absorption rate of insulin, caused by the vasoconstrictor action of the pressor substance.

Griffiths (63) extended the study of anterior pituitary effect upon the insulin content of rat pancreas. In growing normal rats, without any treatment, the insulin content of the pancreas increases in approximate parallelism to the body weight. When rats, 80 gm. in weight, were hypophysectomized, the body weight became stationary, the pancreas atrophied, while its insulin content increased. If hypophysectomized after the body weight had reached or exceeded 100 gm., the rats lost body weight and the pancreatic insulin diminished nearly in proportion with the body weight. Injection of anterior pituitary extracts induced in both groups an increase in the insulin content of the pancreas, which was nearly proportional to the increase in body weight, the latter due to the growth hormone. This evidence indicates that in the growing animal the hypophysis exerts no specific control on the insulin content of the pancreas, but rather that the increase in insulin content is part of "the general increase of constituents involved in somatic growth." This view merits consideration in the interpretation of the earlier findings of Marks & Young (1940) and of other workers, showing that injections of anterior pituitary extract induce for a while a substantial increase in the insulin content of the pancreas of normal rats.

In the absence of the anterior lobe of the hypophysis, epinephrine causes markedly less hyperglycemia in dogs than when the anterior lobe is present. This is true even when a starved but intact dog, whose hepatic glycogen stores are depleted, is compared with a dog that has about 3 per cent liver glycogen but whose anterior lobe has been removed. Bodo and his colleagues (64, 65) attribute to this relationship the extreme insulin sensitivity of hypophysectomized dogs.

Cats can be made permanently diabetic by continued injections of anterior pituitary extract only when partially depancreatized. Lukens & Dohan (66) made the remarkable observation that a course of daily phlorhizin injections restores the carbohydrate tolerance of such diabetic cats to normal. Concomitantly, morphologic restoration of the islands of Langerhans was observed, i.e., the diabetes was cured.

Sex hormones.—Rats, when fed on a high carbohydrate diet, showed a slight, transient glycosuria. After becoming aglycosuric, injections of stilbesterol produced hyperglycemia and glycosuria. In partially depancreatized rats, kept on a constant carbohydrate intake, the symptoms of diabetes were exacerbated by injection of stilbesterol; dehydrostilbesterol, estradiol, and equilin produced similar effects (67). These results are in line with those previously obtained by

Dolin (68) on ferrets by estrogen injections, but in conflict with the findings of Griffiths *et al.* (33).

Evans and his co-workers (69) confirm previous reports [(Griffiths *et al.*, 1940)] to the effect that subcutaneous estrogen implants produce an increase in the insulin content of rat pancreas. Good evidence is offered to show that this action is mediated by the pituitary. The pituitaries of estrinized rats, when implanted in untreated rats, produced the same effect as the estrogen (α -estradiol dipropionate) itself, while hypophysectomized rats showed no response at all to the estrogen. Increase in the insulin content of the pancreas under the influence of estradiol and of stilbesterol was observed by Funk *et al.* (70), who attribute a similar effect to a protein fraction of the anterior pituitary.

Euler *et al.* (71) observed a decrease in blood pyruvic acid of rats in estrus, three to four hours after the injection of testosterone propionate. This substance and estrone greatly depress the abnormally high pyruvate content in the blood of rats with Jensen sarcoma.

Adrenals.—Ingle & Thorn (72), examining the effects of 11-desoxycorticosterone (*A*) and 17-hydroxy-11-dehydrocorticosterone (*B*) on rats, found great differences in the influence of these substances upon the flow of carbohydrates in the organism. One group of the rats was subtotally depancreatized, another group adrenalectomized in addition. Slight glycosuria occurred in part of the first group, while all of the rats in the second group were aglycosuric. Injection of *A* permitted further growth of all members of both groups and caused no glycosuria, whereas *B* caused marked degrees of glycosuria and in some instances ketonuria, increased nitrogen excretion, loss of body weight, dehydration, and in most cases death. Carbon dioxide combining values of the blood plasma suggest that acidosis, due to ketosis, may have been the fatal factor. Ingle (73) reported that 17-hydroxy-11-dehydrocorticosterone exerts a marked glycosuric effect also in the normal intact rat. More recent experiments on mice lend further support to the view that the anterior pituitary exerts its antagonistic effect to insulin by mediation of the adrenal cortex (74).

METABOLISM OF KETONE BODIES

Concepts regarding the metabolism of ketone bodies have undergone radical changes during the past decade. "Fats burn only in the fire of carbohydrate" proved to be a catch phrase, invalidated by much conclusive evidence showing that the "ketolytic" effect of carbo-

hydrates is nonexistent. Ample proof has accumulated to show: (a) that β -hydroxybutyric and acetoacetic acids are normal metabolic products; (b) that the liver utilizes very little if any of these ketone bodies, while muscle tissues are capable of utilizing considerable amounts of them as a source of energy; (c) that carbohydrate is the preferential source of energy of the liver, in lack of which the liver is compelled to consume increased amounts of ketone-yielding metabolites (fats, amino acids). When, as a consequence, the liver produces ketone bodies at a rate that exceeds the rate of peripheral utilization, hyperketonemia and ketonuria ensue. Deglycogenation of the liver consistently causes ketosis. The action of carbohydrates is only anti-ketogenic in that they furnish to the liver its preferential fuel and obviate the consumption of large amounts of ketogenic metabolites.

Recent contributions to the subject uniformly bear out these points. Barnes *et al.* (75) have shown that the muscle of rabbit, dog, goat, and man utilizes appreciable amounts of ketone bodies. Harrison & Long (76) found this to be the case in rats, and Crandall and his colleagues in dogs (77). The capacity of the muscle tissues to utilize ketone bodies varies with the species but it increases in all instances with increasing concentrations of the ketone bodies present in the blood stream. Friedemann (1936) was the first worker to demonstrate this fact on the dog; Wick & Drury (78) have now proved it to hold true for the rabbit, and Nelson *et al.* (79) for the rat. In the intact rabbit, β -hydroxybutyric acid is utilized until this substance accounts for about 90 per cent of the oxygen consumption of the animal; in the rat the rate of utilization increases until the concentration of ketone bodies in the blood reaches about 10 mM. Koehler *et al.* (80) concluded from experiments with human subjects that neither glucose nor insulin exerts any ketolytic effect, but that their action consists exclusively in the suppression of ketone production in the liver (anti-ketogenic action); this is in line with numerous previous observations on laboratory animals.

In fasting human subjects hunger ketosis decreases during exercise and increases again during rest following the exercise (81). The phenomenon was also clearly demonstrated on rats. The secondary increase of ketosis after exercise does not occur in the isolated muscle (Blixenkronne-Møller); therefore, in the intact animal, the increment must originate from the liver as a result of a progressive deglycogenation of this organ during exercise.

MacKay and his colleagues found that acidosis (82) and dehydra-

tion (83) exert antiketogenic action in the fasting rat. The antiketogenic action of acidosis induced by hydrochloric acid feeding may be correlated with an increase in the hepatic glycogen stores, derived from proteins. During dehydration the fall in ketonemia is likewise connected with increase in liver glycogen, probably of protein origin. Sodium bicarbonate acts in the opposite direction, i.e., it increases ketosis and decreases liver glycogen content.

As to the further catabolism of acetoacetic acid, Lehninger (84) joined those who consider its hydrolysis to acetic acid as a possible step. This author claims to have observed in minced muscle, and also in cell-free muscle and kidney extracts, an enzymatic hydrolysis of acetoacetic acid to acetic acid. Stadie *et al.* (85) found in liver slices a complete lack of conversion of ketone acids to acetic acid. Nor was acetoacetic acid converted to carbohydrate by liver slices from diabetic cats (86), whereas these slices retained their ability to convert *D*-lactate into carbohydrate. This evidence, the authors state, contradicts the hypothesis that fatty acids are converted into glucose in the diabetic liver.

Mirsky *et al.* (87) observed that ketosis resulting from the administration of phlorhizin develops more readily in many diabetic patients than in healthy subjects. Since ketosis is a function of the deglycogenation of the liver, the degree of ketosis that occurs under the effect of phlorhizin is regarded as an index of the ability of the liver to store and retain glycogen. In children this ability is markedly less than in adults, as shown by the fact that healthy as well as diabetic children lose, under the effect of phlorhizin, almost twice as much liver glycogen per kilo body weight as do adults. The difference is marked, in that 36 per cent of the healthy children developed ketosis under the same standardized conditions which failed altogether to produce ketosis in healthy adults.

Somogyi (88) studied the effect of insulin upon the production of ketone bodies in normal and diabetic men and dogs. Quite generally insulin causes an initial drop of the ketonemic level, but when protracted hypoglycemic states are produced by the insulin, ketosis increases. Insulin hypoglycemiae cause exacerbation of diabetes primarily by increasing the rate of hepatic glycogenolysis, often manifested in subsequent hyperglycemia and glycosuria (Somogyi, 1938), and secondarily by inducing ketosis (or by increasing an existing ketosis) through the deglycogenation of the liver. Somogyi and his associates and Lavietes & Peters (89) applied these findings to the

treatment of diabetic patients with gratifying results. It is of theoretical as well as of clinical significance that an increase in the rate of hepatic glycogenolysis invariably leads to increased ketonemia, irrespective of the factor that is responsible for the acceleration in glycogen breakdown. Thus Somogyi & Kirstein (90) observed the occurrence of ketosis in individuals subjected to artificial hyperthermia. Incidentally, Loseke & Gunderson (91), studying the effect of artificial hyperthermia on rabbits, found a considerable decrease in the glycogen content of the liver.

Several reviews of carbohydrate metabolism have appeared within the past year (92 to 95).

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THE JEWISH HOSPITAL OF ST. LOUIS
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FAT METABOLISM

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The present review covers only a part of the literature, since the majority of foreign journals have become unavailable in the past year. In a few cases the summaries published in *Chemical Abstracts* could be used, and this has been indicated in the bibliography. A considerable number of foreign publications had to be omitted.

The present period in biochemistry is characterized by an increased emphasis on new tools rather than on definitive results. The possibilities of the application of stable and radioactive isotopes, of the ultracentrifuge, the electrophoresis apparatus, etc., have occupied the attention of a great number of workers. It is perhaps indicative of the present trend that the lipid metabolism in plants, unquestionably a fascinating and important subject in comparative biochemistry, is practically not mentioned in this review.

GLYCERIDES AND FATTY ACIDS

Absorption.—Winter & Crandall (1) were unable to demonstrate the direct portal absorption of fatty acids in normal unanesthetized dogs. No significant differences between the fatty acid contents of portal and arterial blood (obtained by the angiotomy technique) were found in samples taken before and during fat absorption. A similar conclusion was reached by Little & Robinson (2).

Burr and co-workers (3) studied the absorption and transport of the methyl esters of fatty acids across the intestinal mucosa in adult rats. The acids were characterized by their content of spectroscopically distinguishable conjugated compounds produced by partial isomerization with alkali of the corn oil fatty acids. Both the neutral fats and the phospholipids of the mucosa were examined. In the first half hour of absorption more than 50 per cent of the acids of the neutral fat had exchanged, in contrast to only 3 per cent of the phosphatide fatty acids. The amount of tagged fatty acids in the neutral fat remained fairly constant between one-half hour and eight hours after absorption; the entry of fatty acids into the phospholipid fraction took place gradually and reached a maximum eight hours after absorption.

The authors found it difficult to reconcile this discrepancy with the conception that fatty acids are transported by means of the phospholipids. However, it should be pointed out that the methyl esters of the conjugated corn oil acids appear to be absorbed at a rate different from that of unchanged corn oil. While phosphorylation of a glyceride may not be the only or even the principal mode of transport of the fatty acids in the organism, the validity of this conception cannot yet be considered disproved.

The effect of a deficiency of the essential fatty acids on fat absorption in rats was studied by Burr and co-workers (4). Using the technique mentioned in the preceding paragraph, they observed a slight decrease in the incorporation of fatty acids into the neutral fats and the phospholipids of the intestinal mucosa in fat-deficient rats. Another consequence of the dietary deficiency of the essential fatty acids was discussed by Sinclair & Fassina (5) who showed that rats raised on a high-carbohydrate, low-fat diet absorbed glucose at an excessive rate.

The function of the adrenals in fat absorption formed the subject of two publications. Burr and collaborators (6), again using spectroscopically characterized conjugated acids, found that adrenalectomy in rats, maintained with salt, produced no change in the normal rate of absorption of corn oil fatty acids. According to Bavetta *et al.* (7), untreated, adrenalectomized rats showed a decrease in fat absorption by about 38 per cent. The depression was somewhat less marked on administration of salt and completely abolished by cortin. The decreased absorption is interpreted as resulting from a failure to remove fatty acids at a normal rate, as evidenced by an increased accumulation, after adrenalectomy, of free fatty acids in the intestine.

Deuel and co-workers (8) found the rate of absorption of the lower fatty acids by the rat to decrease in the following order: (a) butyric, caproic, and caprylic acids; (b) nonylic acid; (c) acetic, propionic, valeric, and heptonic acids; (d) capric, undecylic, and tridecylic acids; (e) lauric acid. There also were studies on enhanced absorption of neutral fats by the admixture of sodium glycerophosphate (9) and on disturbed fat absorption in hypophyseal insufficiency (10).

Utilization.—The soybean oxidase which converts unsaturated fats into peroxides was found to oxidize directly only those compounds

$$\begin{array}{c} \text{H} \quad \text{H} \\ | \quad | \\ -\text{C} = \text{C}(\text{CH}_2)_7\text{C}(\text{O})- \end{array}$$

which contain the grouping —C = C(CH₂)₇C(O)— with *cis*-configuration (11). The fatty acid dehydrogenase present in rat and

rabbit organs was reported to dehydrogenate distearin and ethyl palmitate. Hexadecane-1,16-dicarboxylic acid and the lower dicarboxylic acids were not attacked (12). Preliminary evidence points to the conversion of fatty acids into carbohydrate by rat liver slices (13).

An interesting study by Fischer & Bielig (14) of the hydrogenation of unsaturated substances in the animal organism is of importance for the question of fatty acid anabolism. These authors found that hydrogenation was limited to double bonds which were in α,β -position to a carboxyl, carbonyl, or hydroxyl group. The conversion of unsaturated acids to saturated acids and vice versa in the animal body, which involves double bonds separated from the carboxyl group by at least eight carbon atoms, would, therefore, have to be regarded not as a simple addition or removal of hydrogen but as a more complicated process comprising a series of degradations and resyntheses.

Indications for the role of δ -oxidation in the production of β -hydroxybutyric acid from dietary caproic acid were obtained by Morehouse & Deuel (15). The utilization by rats for growth and depot fat formation of a synthetic fat consisting exclusively of acids with an uneven number of carbon atoms was investigated by Appel *et al.* (16). Hydrogenated and ordinary coconut fats were likewise examined. All three fats were utilized equally well; there were no differences in absorption and in the iodine values of the fat depots. With regard to the last finding it should, however, be pointed out that coconut fat, which normally contains almost 92 per cent of its fatty acids as saturated acids may not be particularly appropriate for experiments of this type. The feeding to men and dogs of the fat consisting of acids with an uneven number of carbon atoms resulted in the secretion of small amounts of pimelic acid, whereas the hydrogenated coconut fat produced sebacic acid (17).

Emmrich & Emmrich-Glaser (18), in confirmation of the well-known results of Verkade and collaborators, found in feeding experiments with higher even-numbered saturated dicarboxylic acids that the utilization of these by the organism increased with increasing chain length of the acid. The C_{16} and C_{18} acids were almost completely oxidized. The alkyl derivatives of malonic, succinic, and glutaric acids were oxidized in the dog with much greater difficulty than the corresponding normal dicarboxylic acids (19).

Schantz *et al.* (20) studied the effect of the addition of egg phospholipids to certain vegetable oils on the nutritive value of the fats for growing rats. Richter (21) examined the nutritive value of nine

different fats by the single food choice method. The longest survival time of rats (fifty-three days) was noted with butter fat. Lavik & Baumann (22) reported a curious effect of dietary fat on tumor formation. The addition of 15 per cent of fat to the diet of mice treated with limited amounts of 20-methylcholanthrene increased the tumor incidence from 12 to 83 per cent. The activity of the fat was found to reside in the fatty acid fraction, ethyl laurate being as effective as the natural fats.

Deposition.—McHenry & Gavin (23) continued their interesting studies of the effect of the B vitamins on fat synthesis. For the synthesis of fat from protein by the rat a supply of pyridoxin and thiamin is needed. Thiamin alone, while ineffective in this case, suffices for the synthesis of fat from carbohydrate. The fatty acids synthesized by rats which received a high-carbohydrate, fat-free diet together with thiamin, riboflavin, pyridoxin, and choline were largely C_{16} and C_{18} acids. The C_{18} and the unsaturated acids were relatively increased when the liver fraction (biotin) which produces fatty livers was administered (24).

Winter *et al.* (25) reported experiments with Eck-fistula dogs which again emphasized the importance of the liver for the formation of fats in the body. The values for total fatty acids and cholesterol in the serum of these animals were considerably lower than in normal dogs, particularly in the later stages of developing liver insufficiency. The animals exhibited a progressive loss of body fat without increased fat loss in the feces. This was obviously due to the failure of the body to synthesize sufficient amounts of fat. It was not possible to produce alimentary hyperlipemia in dogs with Eck fistulas.

The possible mechanisms of fat synthesis from sugar solutions by cultures of *Endomyces vernalis* were reviewed by Reichel (26).

Ketosis.—Publications on ketosis were quite numerous in the past year. No attempt will be made here to give a complete survey of the field, except to point out a few results which are directly related to the main object of this article. The biological fate of 1,2-dihydroxybutyric acid was investigated by Hoff-Jørgensen (27). *dl*-Erythrodihydroxybutyric acid, when intravenously injected into dogs, was destroyed as rapidly as *l*(+)-lactic acid. The *d*-threo acid was oxidized more slowly; the corresponding *l*-acid was practically not attacked. Both *dl*-erythro- and *d*-threodihydroxybutyric acids were antiketogenic in dogs with phlorhizin diabetes.

Valeric, heptylic, pelargonic, and undecylic acids were ketogenic in

rabbits (28). With the exception of α -methylcaproic acid, only those branched-chain fatty acids which carried the methyl or ethyl side chain in another than the α -position appeared to give rise to ketone bodies (29). The relationship between the diet and subsequent fasting ketonuria in rats formed the subject of two papers (30, 31).

Stadie *et al.* (32), in continuation of their interesting studies of fat metabolism in diabetes, investigated the formation of fermentable carbohydrate from acetoacetate by kidney slices from normal and depancreatized cats. In contrast to previous claims (33), no formation of carbohydrate in normal rats and cats and in diabetic cats could be detected. There was no evidence for the conversion of fatty acids into carbohydrates by liver slices from diabetic cats. The same authors (34) were able to adduce additional corroborative evidence for the hypothesis of multiple alternate oxidation of fatty acids in the liver, according to which the fatty acid along its entire chain is simultaneously oxidized at alternate carbon atoms with complete conversion to ketone bodies. Lipocaic deficiency, in contrast to insulin deficiency, produces no increase in blood ketone bodies of depancreatized dogs (35). The hyperketonemia of insulin deficiency is not influenced by lipocaic, and the mobilization of liver fat by lipocaic is not accompanied by an increase in the blood ketones.

Glycerol ethers.—In view of the isolation by Holmes and collaborators (36) of batyl alcohol ($C_{21}H_{44}O_3$) from the unsaponifiable fraction of yellow bone marrow of cattle, a recent suggestion as to the biological origin of the glycerol ethers may be of interest. Baer & Fischer (37) consider it possible that the acetal phosphatides which are known to be constituents of the living cell (38) are converted to the corresponding ethers by reductive splitting of the carbon-oxygen bond of the acetal and subsequent removal of the phosphoric acid. It is, in the opinion of the reviewer, not impossible that these glycerol ethers may occur in nature as esters with fatty acids or phosphoric acid.

PHOSPHATIDES

Chemical constitution.—Folch & Schneider (39) reported that between 40 and 70 per cent of the cephalin fraction from cattle brain consisted of a substance which contained the hydroxyamino acid serine in ester linkage with a phosphatidic acid. Both the amino and carboxyl groups of the amino acid were free in this compound, for which the name phosphatidyl serine was proposed. Serine was isolated as the

free amino acid (40) and as the β -naphthalenesulfonyl derivative (41). The discovery of this serine cephalin, which may be of great importance for a better understanding of the intermediary phosphatide metabolism, confirms in a beautiful manner the old findings of MacArthur (42), to which unfortunately not enough attention had been paid. The conception of the presence of an amino acid in the cephalin fraction is in good agreement with the strongly acidic properties of cephalin emphasized in recent papers from this laboratory (43, 44, 45).

In a procedure which may be useful for the exploration of the structure of various phosphatides the isotope dilution method was applied to the study of the distribution of choline and ethanolamine in pig heart and liver phosphatides (46). The pure bases containing a known amount of the N^{15} isotope were added to the phosphatide hydrolysis mixtures, and the isotope dilution which had taken place was subsequently determined in pure specimens of the bases isolated (choline as the mercuric chloride double salt, ethanolamine as a new derivative: the 3,5-diiodosalicylate). Blix (47) pointed out that choline-free cephalin from cattle brain contained only 25 to 50 per cent of ethanolamine cephalin.

Lesuk & Anderson (48) reported an interesting observation, viz., the isolation from the lipids of *Cysticercus fasciolaris* larvae of a saturated cerebroside (dihydrophrenosin) and of a hydrolecithin (dipalmityl lecithin).

Formation and turnover.—The distinction between metabolic and nonmetabolic phospholipids, so often made in the past, is losing much of its sharpness. With our growing insight into the enormous rapidity of regeneration of tissue constituents the conception of metabolically inert body deposits or structural elements will disappear. As a recent example the rapid uptake of radioactive phosphorus by nuclei may be mentioned (49). Most of the work on these problems is based on the use of isotopes as labels. For this reason, a very important paper by Hevesy & Hahn (50) should be mentioned first which, although published in 1940, appears not yet to have been sufficiently noticed. In this investigation the rates of uptake of radioactive phosphorus by the individual phospholipids (lecithin, cephalin, sphingomyelin) in different organs of rabbits, rats, hens, and frogs were compared with the specific activities of the inorganic phosphorus present in the same organs. A satisfactory measure of the synthetic capacities of various organs was thus obtained which in its qualitative aspects did not materially differ from results reported

previously. The intestinal mucosa and the liver were the most active organs, followed by the kidney and muscle. It had previously been shown that twenty-four hours after the administration of radioactive phosphate considerably more labeled lecithin than cephalin was found in rat liver and intestine (51, 52). This observation was confirmed by Hevesy & Hahn, but they found this order to be reversed in experiments of short duration (four hours). The activity of lecithin showed an almost linear increase with time; the activity of cephalin increased rapidly in the first four hours and then abruptly became almost stationary. The two curves intersected at about twelve hours after the administration of the radioactive phosphorus. The simplest explanation of this phenomenon is perhaps the assumption that the liver cephalin fraction contains a component in a concentration of about 25 per cent which is renewed at a considerably higher rate than the bulk of the cephalin fraction. It is, on the basis of findings soon to be published from this laboratory, quite possible that this "fast" cephalin is identical with the serine phosphatide described by Folch & Schneider (39). An interesting observation concerning sphingomyelin should also be mentioned. In experiments of short duration this phosphatide was found to be renewed in the muscle at a rate intermediate between that of cephalin and lecithin, whereas it proved the most active phosphatide fraction at the end of nine days.

Fries & Chaikoff (53) found that large variations in the amount of radioactive phosphate injected into rats and mice produced no change in the amount of radioactive phosphorus recovered in blood and brain. The same authors (54) determined the recovery of subcutaneously administered radioactive phosphorus in various parts of the brain of rats of various age groups. The phosphorus deposition which was not uniform in the different parts of the brain was highest in newborn animals. Waelsch *et al.* (55), using deuterium as indicator, studied the deposition of brain lipids (characterized by the fatty acid and unsaponifiable fractions) in young rats maintained with a constant concentration of heavy water (D_2O) in their body fluids. They attempted to distinguish between the influences of growth and of myelination on the rate of deposition of brain lipids and found the first to be the more important factor.

Other studies in which radioactive phosphorus was applied as indicator comprised the demonstration of a concurrent synthesis and destruction of phospholipids in rat liver slices (56), evidence of an increased deposition of newly formed phosphatides in denervated

muscles (57, 58), and a study of the rate of formation of sphingomyelin in various tissues of young cats (59). Haven (60) found a more rapid turnover of lecithin than of cephalin in rat carcinosarcoma. The rate of formation of sphingomyelin in the same sarcoma resembled that of cephalin (61). An investigation by Tuttle *et al.* (62, 63) of the rate of synthesis of the nucleoprotein, phospholipid, and acid-soluble fractions in the organs of normal and leukemic mice showed a considerable increase in the uptake of small doses of radioactive phosphorus by the nucleoprotein and acid-soluble fractions of liver, spleen, and lymph nodes in leukemic as compared with normal animals. The phosphatide metabolism of spleen and lymph nodes was only slightly changed, while in the liver a depression of phosphatide formation was noted in leukemic mice on the first day after phosphate administration.

Tissue lipids.—Klenk & Rennkamp (64) described a method for the preparation from brain of pure sphingomyelin, free from glycerol-containing substances. The deposition of cholesterol and phospholipids in various parts of the central nervous system of rats (65) and rabbits (66) of various age groups was studied. Knouff *et al.* (67) examined the effect of excessive muscular activity on the adrenal lipids of the guinea pig. A considerable fall in the cholesterol content of the adrenal gland but no corresponding decrease in the amount of total lipids was observed. Oleson & Bloor (68) reported that fasting produced a slight increase in the phospholipid and a decrease in the cholesterol content of the adrenal glands of guinea pigs. The adrenal lipids of rabbits were likewise examined (69).

Lipidoses.—Klenk (70) has continued his fundamental work on the chemistry of the lipidoses. The new lipid with a high sugar content which had been previously isolated from the brain cerebrosides in a case of Tay-Sachs' disease (71) was again observed. It contained 1.0 per cent phosphorus, 3.3 per cent nitrogen, and 23.6 per cent galactose. The same lipid, although in much smaller concentration, appears to form part of the cerebroside fraction of normal brain. Klenk (72) prepared the lipid from large amounts of normal brain extracts in a yield of 10 to 14 per cent of the total cerebrosides. It was found to consist of fatty acids, mainly stearic acid, sphingosine, galactose, and a new nitrogen-containing organic acid which was designated neuraminic acid. This extremely unstable amino acid had either the formula $C_{11}H_{21}NO_9$ or $C_{10}H_{19}NO_8$. It did not contain or yield a hexosamine.

The recent report of Halliday *et al.* (73), that the cerebrosides de-

posited in the spleen in Gaucher's disease contained glucose instead of the galactose ordinarily present, was confirmed by Klenk & Schumann (70). The latter found the spleen cerebroside to consist of 49 per cent behenyl sphingosine glucoside and 39 per cent lignoceryl sphingosine glucoside. The cerebroside isolated from the brain in the same case of Gaucher's disease had, however, the normal composition and contained galactose.

Ferraro & Jervis (74) confirmed the observation of Beumer & Gruber (75) that the general morphological picture of Niemann-Pick disease could be produced experimentally by intravenous injection of a sphingomyelin emulsion. Other papers which might be mentioned include the chemical analysis of a xanthomatous tumor with regard to its cholesterol and phospholipid content (76) and a study of necrobiosis lipoidica diabetorum (77).

Lipoproteins.—Complexes between lipids and proteins are of biological importance. They are widely distributed in living matter, occurring in cell nuclei, mitochondria, cell membranes, chloroplasts, in egg yolk, and in milk. It is in combination with proteins that the phospholipids appear to be transported in blood. Most of the heavy protein fractions isolated from tissues, the thromboplastic factor, many bacterial antigens, and viruses contain lipid-protein complexes. Several studies of substances of this nature have appeared in the past year. In connection with x-ray diffraction studies on the structure of the nerve myelin sheath (78), Schmitt and co-workers investigated the x-ray diffraction patterns obtained with purified lipids (79) and lipid emulsions (80). Palmer *et al.* (81) reported x-ray diffraction studies of synthetic lipoproteins containing cephalin, viz., cephalin-histone and cephalin-globin complexes (44). In these complexes the protein appears to occur as thin layers inserted between the bimolecular leaflets of cephalin.

A number of studies dealt with the thromboplastic protein from lungs, a lipoprotein of extremely high molecular weight which has the ability to effect or catalyze the conversion of prothrombin to thrombin. Certain cephalin fractions, it will be recalled, also exhibit this thromboplastic activity. A study of the effect on prothrombin of a radioactive thromboplastic protein preparation, which was isolated from rats to which radioactive phosphorus had been administered, showed that very small, although possibly significant, amounts of phosphorus-containing substances were transferred to the thrombin (82). Methods for the isolation of the thromboplastic protein from

beef lungs and some of the electrophoretic and immunological properties of this lipoprotein were also discussed (83). It is noteworthy that heparin was found to be able to displace the phosphatide constituents of this substance with the formation of a heparin-protein complex of markedly anticoagulant properties (84). The phosphatides contained in the thromboplastic protein were subjected to chemical analysis (85). The lecithin and cephalin fractions both showed thromboplastic activity, whereas the sphingomyelin fraction proved inactive. It is remarkable that the complex phosphatide mixture, comprising at least three phosphatides of different electrophoretic properties, migrated uniformly when bound in the thromboplastic protein. The phospholipoprotein character of this agent was confirmed in a more detailed study in which its purification by means of electrophoresis was described (86).

Oxidation.—Rusch & Kline (87) described the catalytic influence of glutathione, cysteine, ascorbic acid, thiamin, riboflavin, pyridoxin, and methylene blue on the oxidation of crude phosphatide preparations from egg yolk and rat liver. A number of compounds, among them several carcinogenic agents, inhibited the oxidation. The catalytic effect of ascorbic acid on the oxidation of rat liver phosphatides which takes place at pH 4 was investigated in greater detail by Deutsch *et al.* (88). Hydroquinone and a number of carcinogenic substances, of which aminoazotoluene and benzpyrene were most active, inhibited the oxidation of this system. The experimental results are open to some criticism; e.g., no chemical analyses of the phospholipids used as substrates are reported. The acid reaction of the oxidation system could, especially in experiments of long duration, have given rise to hydrolysis of the phosphatides. Control experiments on the oxidation of glycerophosphoric acid would, therefore, have been of interest. Peters & Cunningham (89) found the oxidation of lecithin in presence of glutathione to be somewhat increased by the addition of diphtheria toxin. According to Lardy & Phillips (90) the rate and duration of oxygen consumption of bull spermatozoa in glucose-free media was greatly increased in the presence of phospholipids. Ascorbic acid had no effect on this system.

LIVER

Influence of external factors.—The work to be discussed in this section is so intimately linked with problems of carbohydrate and protein metabolism, transmethylation, nutrition, and the function of

the vitamin B group, that only by a highly arbitrary selection of material can too much overlapping with other chapters of this *Review* be avoided. Investigations of problems related to the fatty infiltration of the liver were actively continued in the past year. Ralli *et al.* (91, 92) studied the lipid distribution in normal human livers and in cases of cirrhosis and fatty infiltration. A study of the relationship between carcass lipids and liver lipids in the fasting three-months-old mouse was carried out by Hodge *et al.* (93). During two days fasting 80 per cent of the total body lipids were found to disappear; the remaining 20 per cent were only slightly reduced by continued fasting. The total lipids of the liver increased two to three times in the first day of fasting, returned to normal on the second day, and decreased to about half of the original content on the third and fourth days of fasting. The liver fraction most affected by these changes was the neutral fat fraction. The absolute concentration of the liver phospholipids remained practically constant. It should be of interest to examine the rate of turnover of the phospholipids under the conditions of the experiment, since the swamping of the liver with fatty acids is likely to exert a pronounced effect. It is known that in fatty livers produced by partial hepatectomy the formation of phosphatides is impeded (52). The fatty changes produced by fasting in another organ, viz., the kidney, were shown to be due to the infiltration of depot fat into the medulla (94).

Chaikoff and collaborators (95, 96) submitted new evidence in support of their hypothesis that the influence of the pancreas on lipid metabolism was exerted through a factor present in the external secretion of the pancreas. They reported that the formation of fatty livers due to pancreatectomy or duct ligation in dogs maintained with insulin could be prevented by the injection of fresh pancreatic juice. It should be noted that in these experiments the dogs had received raw pancreas for a considerable period preceding the administration of pancreatic juice. The comparison of the lipotropic effects of choline and pancreas preparations in depancreatized dogs maintained with insulin showed that fatty livers were prevented by the daily administration of 36 mg. of choline or 5.5 gm. of raw pancreas per kilogram of body weight (97). The choline content of the pancreas fractions was not directly determined, but merely estimated to be much less than the minimum required dose of choline.

Several papers which in a general way confirmed Dragstedt's view on the lipocaic factor of pancreas appeared during the past year (98,

99, 100). The agent contained in pancreas was found to be labile with respect to heat (97, 100).

The influence of the adrenals on the transport of fat into the liver was studied by Burr and collaborators (101) by means of labeled fatty acids, as outlined in the section on glyceride metabolism. Adrenalectomized rats maintained with salt showed an impaired ability to deposit absorbed fatty acids in the neutral fat fraction of the liver. This condition responded to the administration of adrenal cortex extract. It is noteworthy that the influence of the adrenals was exerted only on the neutral fat fraction. The typical sex difference in liver fat, i.e., the larger amount of fat found in the livers of females, appears to be due solely to the acetone-soluble fat fraction.

The removal of the spleen in rabbits poisoned with phosphorus was stated to inhibit the fatty infiltration of the liver (102). Dryerre & Robertson (103) observed that by the end of the fourth month of pregnancy the fatty infiltration of the liver in pregnant ewes reached its maximum. This increase was mainly due to the neutral fat fraction. The phospholipid and cholesterol levels in the livers of mice were depressed when the metabolic rate was increased by the injection of 2,4-dinitrophenol (104).

The work of Gavin & McHenry on the influence of dietary factors on the deposition of fat in the organism probably represents one of the most fruitful approaches to this problem. In a brief note (105) these authors reported certain similarities between the lipotropic effects of inositol and of lipocaic. Inositol prevented the formation of the "biotin" type of fatty liver in rats which can be produced by supplying the animals with biotin together with thiamin, riboflavin, pantothenic acid, and pyridoxin. It could be shown that the liver fraction which produced fatty livers in rats owed its activity to its biotin content (106). The feeding of raw egg white, as well as of lipocaic or inositol, prevented the action of biotin. With regard to the lipotropic effect of inositol, it might be pointed out, although it possibly is of no more than incidental interest, that the presence of inositolphosphoric acid in a phospholipid preparation, viz., soybean phosphatides, actually has been demonstrated (107). The liver fraction which produces fatty livers was studied in rabbits by Leites *et al.* (108).

Singal & Eckstein (109) investigated the lipotropic action of the sulfur-containing amino acids and their derivatives in mice that were kept on a diet low in protein and high in fat. Cystine betaine was found to be lipotropic, in contrast to cystine which is known to pro-

duce fatty livers. The S-methyl, ethyl, propyl, and isopropyl derivatives of cysteine did not cause an increase in liver lipids. Cystine disulfoxide behaved like cystine, whereas cysteic acid, pentocystine, hexocystine, and djenkolic were inert. *dl*-Valine, *dl*-leucine, and *dl*-isoleucine were likewise inactive. Methionine sulfoxide, the growth-supporting action of which had previously been demonstrated (110), retained the lipotropic activity of methionine. Dithiodiglycolic acid also was lipotropic.

Choline and the formation of phosphatides.—It appears to be well established that the growing rat is able to synthesize choline. Jacobi *et al.* (111) found the choline content of growing rats kept on a diet free of choline to increase with the weight of the animals. In the course of eight weeks one animal synthesized as much as 76 mg. of choline. The total choline content may actually have been even higher, since the method used for the extraction of choline from the tissues presumably did not permit the estimation of any choline not in lipid combination.

The interrelationship between choline and some of its possible precursors in the body was studied by Stetten (112). He investigated the metabolic fate in adult rats of the following compounds which contained the N^{15} isotope: ethanolamine, choline, glycine, betaine, and ammonia. Choline and ethanolamine were then isolated from the total phosphatides of the animals. The feeding of the ethanolamine resulted in the replacement of at least 28 per cent of the ethanolamine present in the phosphatides by the isotopic base in the course of three days. A portion of the ethanolamine was converted to choline since 11 per cent of the choline bound in the phospholipids was derived from the isotopic ethanolamine. The reverse reaction, viz., the formation of ethanolamine from choline, apparently did not take place under the dietary conditions of the experiment. When choline was fed, 21 per cent of the choline in the phospholipids was replaced by dietary choline. Betaine and glycine were both converted to ethanolamine. The biological source of the methyl groups required for the synthesis of choline in the body was convincingly demonstrated by du Vigneaud and co-workers (113) in experiments which provided direct evidence of the transfer of methyl groups from methionine to chlorine. Deuteriocholine (containing deuterium in the methyl groups only) was isolated from rats to which methionine containing deuterium in the methyl group had been administered. In the diet employed, which was free of choline, methionine appeared to be the only methyl donor.

Creatine and creatinine are unable to mobilize their methyl groups for the biological synthesis of choline (114). These findings permit the obvious inference that the lipotropic action of methionine is due to its ability to provide the methyl groups for the synthesis of choline. The reason for the lipotropic effect of choline itself, however, is by no means clear.

BLOOD

The manner in which the lipids are transported in the blood has long been a matter of interest. Several contributions to this problem can be found in the recent literature. Blix *et al.* (115) by means of electrophoresis studies investigated the question of the transport of lipids by the proteins of human serum. All electrophoretically separated protein fractions (albumin, α -, β -, and γ -globulins) were found to contain cholesterol and phospholipids. The α - and β -globulins were much richer in lipids than the other fractions. β -Globulin, a notoriously unstable protein, contained the lipids in a more coarsely dispersed form than did the other proteins. Additional electrophoretic evidence of the lability of β -globulin may be seen in the fact that the addition of heparin had a disruptive effect on the globulin patterns and brought about the disappearance of the so-called β -globulin disturbance (116). Blix (117) in an electrophoretic study of serum proteins freed from lipids emphasized the importance of the β -globulin fraction as the transporting agent for the lipids and other colloids present in serum. Cholesterol, when added to the serum protein mixture, migrated with the β -globulin. It is of interest that in lipid-free sera the bilirubin was found to shift from the albumin to the β -globulin. According to Frazer (118), the neutral fat particles in plasma or serum which show Brownian movement under dark-ground illumination have a film of globulin at the oil-water interface which serves to stabilize the emulsion. The crystalline albumin from human serum, described by Kendall (119), is associated with a small amount (2 to 3 per cent) of free fatty acids which cannot be removed without preceding denaturation of the protein. There are indications that the surface of the red cell consists largely of strongly acidic phosphatides of the cephalin type (120).

Hevesy & Hahn (121) investigated the rate of penetration of the rabbit plasma phosphatides into various organs. The most rapid rate of penetration was found in the liver, the cells of which appeared highly permeable to phosphatides. Studies of the nature of the plasma

phospholipids still suffer from the inadequacy of the methods available. Artom (122) based his recent determinations on the estimation of the phosphorus and choline contents of human plasma phosphatides and found (in mg. per 100 cc. of plasma) total phospholipids 152 ± 16 , choline phospholipids (lecithin and sphingomyelin) 122 ± 14 , cephalin (by difference) 30 ± 9.5 . No preferential increase in choline phospholipids could be demonstrated in alimentary lipemia. Brante (123) likewise failed to observe a predominant role of choline phosphatides as fat transporting agents in lipemia. Blix (124) concluded, on the basis of glycerol and phosphorus determinations, that the phosphatides of human serum contained about 13 per cent of glycerol-free phosphatides (presumably sphingomyelin). Ramsay & Stewart (125) reported remarkably high sphingomyelin values for blood, viz., an average of 64 per cent of the total phosphatides. There seemed to be somewhat more cephalin than lecithin present, although the extreme variations in the analytical results (lecithin between 0 and 33 per cent, cephalin between 11 and 35 per cent of the total lipids) do not allow final conclusions to be drawn. Singer (126) reported observations on the apparent lysophosphatide content of blood.

Blix (127), assuming the correctness of his sphingomyelin determinations mentioned in the preceding paragraph (124), estimates the triglyceride content of human plasma to lie within the range of 30 to 70 mg. per 100 cc. The composition of the fatty acids contained in the glycerides and cholesterol esters of beef plasma was studied by Kelsey & Longenecker (128). The bulk of saturated acids was contained in the glycerides, whereas 85 per cent of the fatty acids of the cholesterol ester fraction were unsaturated acids with a remarkably high amount (62 per cent) of linoleic acid.

STEROIDS

Wintersteiner & Bergström, in a preliminary note (129) and later in greater detail (130), reported interesting findings concerning the autoxidation products of cholesterol. Colloidal solutions of cholesterol in aqueous sodium stearate, when aerated at 85° , gave rise to 7-keto-cholesterol (in a yield of 40 per cent of the cholesterol used) and smaller amounts of other products, of which 7-keto- $\Delta^{5,6}$ -cholestadiene and 7(α)-hydroxycholesterol were identified. The position 7 in cholesterol, in contrast to the hydroxyl in position 3, appears particularly susceptible to attack by molecular oxygen. 7-Ketocholesterol and 7(α)-hydroxycholesterol may be biological precursors of 7-dehydro-

cholesterol, which is provitamin D₃. It is of interest that cyanide is reported to prevent this autoxidation.

Sperry & Brand (131) continued the study of cholesterol esterase. Their findings seem to speak against the assumption that cholesterol by way of its esters is particularly concerned with the transport of fatty acids. Rat liver emulsions were found to contain an enzyme system which esterified cholesterol. This enzyme appears to be different from the one present in blood serum. No cholesterol esterase was demonstrated in brain.

Treadwell & Eckstein (132) continued their studies of the formation of cholesterol in rats. Four generations, all originating from one group of animals, were used. The effect on sterol production of diets containing 6 and 28 per cent of fat respectively was compared. The cholesterol content of the serum and the neutral fat, phospholipid, and cholesterol contents of the liver were not influenced by the increase in dietary fat. The differences between the fecal and dietary sterols were, however, greater on a high fat diet.

In view of the indispensability of cholesterol for the growth of many insects, the sterol requirements of the larva of the beetle *Dermestes vulpinus* were studied by Fraenkel *et al.* (133). With the exception of cholesterol and its acetate and of 7-dehydrocholesterol and its monobenzoate, all sterols examined were ineffective. Engel *et al.* (134) described a procedure for the isolation of steroids from human urine which will be of value for the study of steroid metabolism. The effect of cholesterol feeding on the lipid distribution in the blood and the organs of rabbits was studied by Weinhouse & Hirsch (135). According to Svendsen (136) the cholesterol content of human serum is a dominant hereditary factor, as shown by the finding of higher serum cholesterol values in twelve out of thirty-four members of the family of a patient suffering from xanthomatosis.

ANALYTICAL PROCEDURES

Trappe (137, 138, 139) contributed a number of studies on the separation of lipids by means of various adsorbents. At the present time, such procedures, although undoubtedly promising, suffer from the fact that the adsorbents used are not yet sufficiently standardized, so as to permit the reproducibility of results. Ramsay & Stewart (125) developed a useful method for the quantitative analysis of blood phospholipids which, by means of the estimation of glycerol, choline, and phosphorus, permits the determination of lecithin, cephalin, and

sphingomyelin in as little as 2 cc. of blood. Blix (124) described a method for the determination of ethanolamine which is based on the volatility of the base at 75 to 80° at a pressure of 10 mm. Hg. Brand & Sperry (140) reported a procedure for the quantitative determination of cerebrosides which makes use of the titration of galactose with ceric sulfate. Another method described recently uses the orcinol reaction for the same purpose (141). The observation that phosphatides are rendered insoluble in petroleum ether by the use of digitonin may be of interest (142). A critical examination of the existing methods for the colorimetric estimation of cholesterol in biological material led Ireland (143) to certain refinements of the usual procedures.

The following reviews will be found of value: a monograph by Thannhauser (144) on lipidoses, and an article by Hirsch (145) on the same subject; articles by Best (146) and by Griffith (147) on choline as a dietary factor; and symposia on intermediary fat metabolism (148), and on the biochemistry of choline (149).

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THE METABOLISM OF PROTEINS AND AMINO ACIDS

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In these unusual times, the task of abstracting scientific contributions to any particular field of biochemistry relates largely to a review of the American literature. Quite probably research activities have been curtailed to a smaller extent in this country than in any other during the prevailing war conditions, but added to this is the fact that European and Asiatic journals have not been received in this country as regularly even as they have been published. Among the articles relating to the subject of this chapter that have been considered by the reviewer in preparing his manuscript, over 80 per cent appeared in American journals. This obviously is not a normal distribution of scientific literature in this field.

THEORIES OF PROTEIN METABOLISM

Perhaps the most significant series of investigations on the metabolism of proteins and amino acids, extending into the current year, has been carried out in this country by a group of scientists headed by Rudolf Schoenheimer, whose presence in this country was more a matter of circumstance than of choice. If political intolerance can be said to have any redeeming features, it would be in the enrichment of the intellectual life of those countries not so afflicted, by the enforced migration of scholars of distinction in their chosen fields of study. Among these scholars, Schoenheimer stood pre-eminent. His tragic death on September 11, 1941, must be recorded with sincere regret.

The investigations of Schoenheimer and colleagues, involving the use of amino acids and other nitrogenous compounds that had been labeled by the inclusion in their molecules at strategic points of the N^{15} isotope and of deuterium, permitted a direct attack upon many problems of intermediary metabolism that previously were unsolvable, or that had been incompletely solved by the accumulation merely of indirect or circumstantial evidence. Urinary creatinine was shown indubitably to be derived from tissue creatine, the major mechanism for urea formation was proven to be the ornithine-arginine cycle, the conversion of phenylalanine to tyrosine was found to occur readily

in the body, even in the presence of an excess of dietary tyrosine, the importance of glutamic and aspartic acids in transamination was established, the conversion of ornithine to proline and glutamic acid was shown to occur, and the anabolic utilization of unnatural isomers by inversion to the natural form was demonstrated with *d*(+)-leucine. Furthermore, new phases in amino acid metabolism were discovered that could only have been suspected previously, such as the irreversibility of the deamination reaction involving lysine, as compared with the ready reversibility of the same reaction with leucine, histidine, and many other amino acids. However, the most important discovery of the researches of Schoenheimer and his colleagues with labeled amino acids was the revelation of the existence of a dynamic equilibrium between the tissues of the body and the surrounding nutrient media that had not previously been suspected.

Schoenheimer & Rittenberg (1) in a recent review of their work, explain the situation in the following words:

The fact that the living organism, in contrast to the dead material, keeps constant the form of the cells and organs as well as the chemical structure of the large molecules has led many investigators to believe that the tissue enzymes, which show their destructive power during autolysis, lie dormant during life and are "activated" only when their function is required. The results obtained with isotopes make such a supposition unnecessary. The experiments indicate that all reactions, for which specific enzymes and substrates exist in the animal, are carried out continually.

Speaking of the many interconversion reactions divulged by the isotope method, Moss & Schoenheimer (2) believe that they cannot be regarded

merely as steps in metabolic degradation. They seem rather to represent automatic and noninterruptable biochemical processes, of synthesis as well as degradation, which are balanced by an unknown regulatory mechanism so that the total amount of the body material and its composition do not change.

The results of these important investigations have been interpreted by Schoenheimer and his colleagues as being inconsistent with the distinction, originally drawn by Folin, between an exogenous metabolism of dietary protein and amino acids, and an endogenous metabolism of the nitrogenous constituents of the tissues. In one publication (3) it is stated: "It is scarcely possible to reconcile our findings with any theory which requires a distinction between these two types of nitrogen." If the Folin theory, modified in some details by subsequent work but essentially unchanged in its insistence upon a distinc-

tion between these two types of protein (nitrogen) metabolism, is no longer tenable, then we can hardly continue to believe in a maintenance requirement of protein, or at least in any predictable requirement. The beautiful relationships between the endogenous output of nitrogen, the maintenance requirement of protein, and the basal metabolism of energy in animals varying widely in size, that have been revealed by the investigations of Terroine (4) and of Smuts (5), would seem to possess little biological reality. The method of computing the biological value of dietary protein from nitrogen balance data (6) must be considered inherently unsound, since it is based upon the assumption of a constant catabolism of nitrogenous tissue constituents, in spite of clear indications of its validity and the reproducibility of its results (7), even in cases where the intensity of the endogenous catabolism of nitrogen has been seriously disturbed by superimposed biological functions (8).

Contrary to the views of Schoenheimer, there must be some way of harmonizing the facts revealed by his classical investigations and the facts revealed by Folin (9) in his equally epoch-making studies. Evidently Folin's conception of the nature of the exogenous metabolism must be radically modified, since evidently the dietary amino acids, whether or not they are consumed in amounts exceeding current requirements, are incorporated continuously and rapidly into the tissue proteins, and are only in part catabolized for the liberation of their nutritive energy. The situation here is reminiscent of the "continuing metabolism of nitrogen" of Borsook & Keighley (10), for which the authors themselves offered so little supporting evidence. As Schoenheimer believed, this "continuing metabolism" in the adult must be considered as being under the control of an unknown regulatory mechanism so that the total amount of body material and its composition do not change. However, as the level of protein nutrition changes, the amount of protein in the body changes also, and even in the adult it is possible, by high-protein feeding, to induce relatively enormous storages of nitrogen (11). This raises the question of the existence of labile reserve stores of protein (deposit protein), for the reality of which Whipple argues so strongly and so effectively (12).

Concerning the reality of a constant destruction or degradation of nitrogenous tissue constituents, the endogenous catabolism of Folin, there appears to be nothing in Schoenheimer's findings inconsistent with this theory. In fact, the recent studies of creatine metabolism

by his group (13, 14) picture a reaction typical of those that must be involved in the endogenous catabolism. Tissue creatine is very constantly undergoing dehydration to creatinine, which is eliminated from the body as a useless metabolite. It is being as constantly formed, and the rate of its synthesis cannot readily be accelerated by an overabundance of its precursors in the tissues, nor by the administration of amino acids. In the words of the authors (13):

It thus differs in its metabolic aspect from all other biological compounds so far investigated with isotopes. It is continuously synthesized but not significantly degraded. Once formed the creatine molecule proper is not involved in any biochemical reaction in which linkages between carbon and carbon or carbon and nitrogen are broken.

The fact that creatine is derivable from dietary amino acids does not militate against the theory of an independent endogenous tissue metabolism (14), since its catabolism is evidently incidental to its functions as a tissue constituent. The elucidation of the endogenous catabolism might be greatly promoted if similar studies with isotopic nitrogen and hydrogen were carried out with reference to the metabolism of other nitrogenous constituents of the tissues, such as carnosine, whose concentration is kept constant within narrow limits, implying a constant degradation and synthesis. It is conceivable also that certain conjugated tissue proteins are subject to a constant catabolism, similar to the catabolism of blood hemoglobin (15).

The summation of these constant catabolic reactions, involving the nitrogenous constituents of the tissues, may be considered to be the endogenous catabolism of Folin. The reality of these reactions is not negated by any of the facts that Schoenheimer's group has revealed. Their independence of the metabolism of dietary amino acids has been rather clearly established recently (16).

The degradation reactions characteristic of the endogenous metabolism may be differentiated from the hydrolytic, interconversion and transamination reactions so startlingly revealed by studies with isotopes, by their relative constancy, and by the irreversibility of the initial step in the degradation. In order that the speed of the endogenous reactions be independent of the amino acids coming from the alimentary tract, it seems necessary that the synthesis of those tissue constituents involved in these reactions proceed along a different path than their catabolic breakdown, as in the case of muscle creatine (14). The reversible hydrolysis to which much of the protein material of the tissues is evidently subjected continuously would seem to

remove this class of compounds, largely at least, from participation in the endogenous catabolism, a conclusion that has been expressed earlier (17) on other grounds. Some conjugated proteins containing prosthetic groups not subject to reversible degradation may be exceptions to this conclusion.

There is considerable evidence that tissue proteins are not all equally readily catabolized in the body. A labile protein reserve, sometimes called "deposit protein," is suggested by the well-known lag extending over many days in the adjustment of the adult organism to a sudden decrease or increase in the protein supply. Such a reserve is clearly indicated by the evidence secured by Whipple and co-workers (12) of the existence of considerable stores of material from which the body can manufacture new hemoglobin or plasma protein. The evidence adduced by Whipple indicated "that this reserve store is a bulwark against infection and some poisons" (18). When the store is depleted, resistance to infection is lowered (19) and susceptibility to chloroform (20) and arsphenamine (21) poisoning may be greatly lowered. The fasting ketosis of rats is the more severe the lower the protein content of the preceding diet (22), apparently because the prior protein intake determines the amount of reserve protein available for catabolism during fasting. This reserve is a source of antiketogenic material, as indicated by the fact that fasting rats, previously on a high-protein intake, better maintain their liver glycogen and blood sugar, as well as a lower level of ketonemia, than fasting rats on a prior low-protein diet. Adults in nitrogen equilibrium on an adequate diet can be induced to store in their bodies considerable amounts of nitrogen and sulfur by the superimposition of protein foods on their basal diets (11), or by the sparing of the basal protein by the superimposition of carbohydrate or fatty foods (23). These induced nitrogen (or protein) storages are readily catabolized when the basal diet is again resumed.

The mysterious feature about reserve protein stores is that they have not been identified chemically in the tissues (24), although their distribution among the different organs has been revealed (25). However, it must be remembered that the usual methods for protein fractionation may not be sufficiently refined to detect the differences that may exist between dispensable reserve proteins and proteins more intimately involved in protoplasmic activity.

Whipple and his co-workers (26) ascribe to the plasma proteins a peculiar role in protein metabolism. They can be drawn upon readily

under conditions of dietary protein deficit and they are readily replenished by adequate protein nutrition. Hence they are part of the reserve protein supply. Furthermore, plasma protein molecules "can be accepted as such by body cells and recast into specific cell protein without loss of nitrogen." Evidence for the latter point was offered by Howland & Hawkins (27), who showed that phlorhizinized dogs can utilize injected plasma proteins with no loss of nitrogen and no production of excess sugar in the urine. Such evidence supports the theory of Schmidt *et al.* (28) that

synthesis of proteins can take place in the body by the transformation of existing proteins into others without first being broken down into amino acids.

Striking confirmation of these ideas is afforded by the report of Reincke, Williamson & Turner (29) on the utilization of glycoproteins of the blood plasma by the mammary gland. The lactating mammary gland takes up glycoprotein from the blood in the production of milk, but no considerable amount of amino acids. On the other hand, the fibroblasts of bone, maintained under aseptic conditions in a culture medium, can utilize plasma proteins only after enzymic or acid hydrolysis (30).

GENERAL PHASES OF PROTEIN METABOLISM

The endogenous catabolism.—The relationship between the endogenous catabolism of nitrogen and the basal metabolism revealed by the investigations of Terroine (4) and Smuts (5) may be distorted, in the rat at least, by certain conditions prevailing prior to the measurement of these two factors (31). A prior high caloric intake raises the basal metabolism without affecting the output of endogenous nitrogen, while a low prior environmental temperature raises the endogenous output of nitrogen without affecting per se the basal metabolism. Previous studies to the effect that a prior low environmental temperature raises the basal metabolism, probably involved an indirect effect of low temperature in raising caloric intake, the latter being the effective agent. Specific protein inanition, employed in the measurement of the endogenous output of nitrogen, induces, according to French, Routh & Mattill (32), an inordinate accumulation of reserve protein on realimentation, so that in a subsequent period of nitrogen-free feeding a much longer time is required before the endogenous level of nitrogen output is attained. This interpretation of the data would be more convincing if assurance were at hand that the different groups of experi-

mental rats were equally well nourished with reference to energy during the periods of protein inanition.

Deamination.—The intravenous injection of amino acids (alanine and leucine) into dogs, simultaneously with the injection of mineral acid to increase the acid load, induces an excretion of urinary ammonia which, according to Bliss (33), is considerably in excess of that occurring when the acid is injected alone. In studies on the relative ammonia-producing effects of the natural and unnatural isomers of these amino acids, it was found that the unnatural isomers produced a somewhat greater increment in urinary ammonia than the natural isomers, in harmony with the fact that most amino acids of the *d*-series are deaminized much more rapidly by tissue slices than their natural antipodes (34). Handler, Bernheim & Klein (35) have studied the ability of broken cell preparations of rat kidney and liver to oxidatively demethylamine the *N*-methyl derivatives of a number of racemic amino acids, and found that whatever oxidation occurred was brought about by *d*-amino acid oxidase. The differential effects noted led them to conclude that the inability of the tissues of an animal to oxidize a *d*-methylamino acid or a *d*-amino acid may be expected to preclude the possibility of utilization of the latter in place of the natural amino acid in the diet of the growing rat, although the converse is not necessarily true.

Transamination.—Cohen, in co-operation with Hekhuis (36), has continued his studies of the rates of the transamination reactions involving *l*(+)-glutamic acid and *l*(—)-aspartic acid, and has shown that the reaction involving glutamic acid and oxalacetic acid in the production of aspartic acid and α -ketoglutaric acid proceeds at much the fastest rate in all tissues studied, the rate of its reversal being considerably slower. The rate of formation of alanine from pyruvic acid by transamination was much slower yet. While the exact role of transamination in intermediary metabolism is not yet clear, the evidence obtained suggests to the authors that the most rapid reaction plays an important metabolic role, involving as it does substrates which act as respiratory mediators in some of the tissues studied. Ågren (37) has shown that α -keto groups in peptide linkage to amino acids can function as acceptors of the amino group from glutamic acid with the formation of dipeptides.

Catabolic stimuli.—Water hunger of such degree that dehydration results has been repeatedly shown to induce an acceleration in the rate of protein catabolism. MacKay, Wick & Visscher (38) have shown

that this increased protein breakdown in the rat is accompanied by a slower glycogenolysis in the liver and a lower level of ketonemia; the association may be most probably ascribed to the antiketogenic effect of increased protein catabolism, but may be in part due to a general depression of metabolism in the dehydrated animal. Epinephrine will also increase protein catabolism, and protein may be spared by carbohydrate, but only if insulin is available, according to Reid (39). Thus, epinephrine and insulin do not have entirely antagonistic physiological roles despite their opposing actions on blood sugar.

d-Amino acids in tumor proteins.—There has been a continuing interest in the claims of Kögl and his co-workers that the proteins of cancer tissue are distinguished from those of normal tissue by the presence within their molecules of amino acids of unnatural configuration. The claim that *d*-glutamic acid is predominant among the *d*-components of tumor tissue proteins is accounted for, by Kögl, Herken & Erxleben (34), by the fact that it undergoes an extremely slow oxidative deamination by rat kidney and liver slices. They confirm the earlier observation of Krebs that most amino acids of the *d*-series are deaminized by tissue enzymes about ten times more rapidly on the average than their natural isomers, but that for glutamic acid the *l*-form is oxidized two or three times as rapidly as the *d*-form. However, the paper is mainly concerned with a critical discussion, with supporting experimental data, of the failure of Lipmann, Behrens, Kabat & Burk (40) to detect differences in the *d*-amino acid content between tumor and normal tissue proteins. The agency concerned in the incorporation of *d*-amino acids into tumor proteins, if such occurs, is indicated by an observation of Herken & Erxleben (41); this confirms earlier work of Waldschmidt-Leitz & Mayer, as well as others, and is to the effect that carcinomatous blood serum, in contrast to normal serum, possesses the ability to hydrolyze to an appreciable extent chemically and optically pure *d*-leucylglycylglycine, and hence contains *d*-peptidases.

To make "confusion worse confounded" in this controversial subject, Woodward, Reinhart & Dohan (42) report their inability to detect any appreciable amount of *d*(—)-glutamic acid in tumor tissue, while Berger, Johnson & Baumann (43) were unable to establish the presence of *d*-peptidases in sera from rats with Flexner-Jobling carcinoma nor in human sera from cases with gastric cancer, nor to demonstrate the artificial production of any considerable amounts of *d*-peptidases in the blood sera of rats by the injection of a racemic

dipeptide, as had been claimed by Waldschmidt-Leitz and others. The indecisive results of von Euler & Skarżyński (44), who report that both carcinomatous and normal sera can hydrolyze *d*-peptides, the former to a greater extent, and of Abderhalden & Abderhalden (45) that carcinomatous sera only occasionally (in 9 cases out of 41) hydrolyze certain *d*-peptides and certain others not at all, do not tend to clarify the issue. Ordinarily, positive results are conceded greater significance than negative results unless they can be shown to involve faulty technique. Also, in such a controversy as that under discussion, faulty technique might be expected to obscure small differences between experimental specimens rather than to indicate differences that do not exist.

Conversion of protein to fat.—McHenry & Gavin (46) have reported experimental data which purport to show that pyridoxin is essential for the metabolism of protein and specifically for the conversion of protein to fat. The experimental technique involved the supplementation of a basal diet high in protein but devoid of fat, carbohydrate, and the B-vitamins with various combinations of the component members of this complex, the feeding of the various experimental rations ad libitum to growing rats, and the determination of the fatty acid content of the livers and carcasses of the rats thus fed. Only when pyridoxin was fed was the fatty acid content of the carcass appreciably greater than that of the controls, but only in such cases did any growth occur. However, fat deposition is a necessary concomitant of growth and, regardless of the nature of the dietary characteristics that induce variable intakes of food, there will in general be a close correlation between the rate of growth secured and the fat content of the carcasses produced (47), within the range of sub-maximal rates of growth. For this reason, for instance, the supplementation of a ration deficient in sodium or chlorine by sodium chloride might be expected to produce, not only more rapid gains in growing rats (48), but also greater contents of carcass fat, without any implication that either sodium or chlorine is at all intimately involved in fat synthesis. Such technic cannot be expected to yield evidence of a direct and fundamental relationship between any dietary supplement and the synthesis of body fat.

SPECIAL PHASES OF AMINO ACID METABOLISM

Antiketogenesis.—Considerable progress has been made in classifying various amino acids as glycogen formers, and hence possessing

antiketogenic properties, or as nonglycogen formers. Glycine, *dl*-alanine and *l*(+)-alanine have been shown (49) to exert the same anti-ketogenic action in fasting rats, although glycine is definitely slower in its action. Since *d*(—)-alanine was later (50) found to be inferior as a glycogen former to both *dl*-alanine and *l*(+)-alanine, it appears that *d*(—)-alanine is better utilized in this way when combined in the racemic mixture, possibly because the much smaller dosage of the unnatural form, with its slow rate of conversion, can be adequately utilized by the liver with no appreciable wastage in the urine. *dl*-Valine (51), *dl*-threonine and *dl*-allothreonine (52) and *l*(+)-arginine (54) are definitely glycogenetic, while *dl*-isovaline (51) and *dl*-lysine (53, 54) are not, nor is glutaric acid (53)—a possible metabolite of lysine. *l*(—)-Tyrosine, in marked contrast to *dl*-tyrosine, is definitely convertible into glycogen in the liver (55).

The uncertainties in the interpretation of the results of such experiments as those cited above, as compared for example with a technique involving the use of compounds labeled with the nitrogen or hydrogen isotope, are illustrated by the report of Flügge (56), from Bickel's laboratory. In what appear to be very carefully controlled experiments, Flügge compared casein and rice protein with respect to their ability to promote glycogen storage in the liver, obtaining much more favorable results with rice protein than with casein. But these results are not interpreted as indicating a greater conversion of the amino acids of rice protein into glycogen than of the amino acids of casein, but rather as a confirmation of Bickel's theory (57) that vegetable proteins induce a higher glycogen storage in the liver than do animal proteins by reason of the greater action of their derivatives (amino acids ?) on the dietary precursors of glycogen, above all on the dietary carbohydrates.

Glycine.—The metabolism of glutathione has been effectively studied by Waelsch & Rittenberg (58) using glycine labeled with isotopic nitrogen. The introduction of isotopic nitrogen into the glutathione of liver and intestine was much faster than into the proteins of those organs. The rapid metabolism of glutathione thus indicated suggests to the authors that the tripeptide may function as an intermediate between free amino acids and proteins. The inability of the body to "de-ethylate" *N*-ethylglycine, in contrast to its ability to demethylate *N*-methylglycine, as well as a limited conversion of glycolic acid to glycine, was demonstrated by Abbott & Lewis (59) in experiments involving benzoate administration to rabbits.

Glutamic acid.—Leuthardt (60) has studied further the mechanism by which the nitrogen of glutamine is readily transformed into urea by liver tissue; evidence is thus afforded that free ammonia is not an intermediary stage in the transformation. On other grounds also, it is argued that this is an instance of urea formation that does not involve the arginine-ornithine cycle. Experiments reported by Barron and others (61) suggest that the oxidation and utilization of the α -ketoglutaric acid, resulting from the deamination of glutamic acid, are increased in the tissues of avitaminotic rats by the addition of thiamin. Since this effect was produced only when the thiamin was previously incubated with the tissue to induce its phosphorylation, it was concluded that the activating protein of α -ketoglutarate oxidase is, like that of pyruvate oxidase, a diphosphothiamin protein.

Arginine and lysine.—The rapid production of arginine from citrulline and glutamic or aspartic acid by surviving kidney slices (rat and guinea pig) is reported by Borsook & Dubnoff (62). The fact that proline, hydroxyproline, lysine, and ornithine may replace the dicarboxylic acids in this reaction is taken to mean, in conjunction with correlative evidence, their conversion into glutamic, or less likely into aspartic, acid. This synthesis of arginine seems to involve an oxidative step in the reaction mechanism, since it is inhibited by potassium cyanide and arsenic oxides. However, no definite evidence of the conversion of lysine to glutamic or aspartic acid was obtained in the experiments of Weissman & Schoenheimer (63) in which rats were fed lysine labeled with both isotopic nitrogen and deuterium. These experiments reveal the peculiar inertness of lysine toward the acceptance of nitrogen from other amino acids, and indicate that the process of biological deamination of lysine is, for reasons that are not clear, irreversible. This unique characteristic of lysine is in harmony with the fact that the α -hydroxy analogue of lysine cannot substitute for the amino acid in promoting growth in rats on a lysine-free diet (64).

Phenylalanine and tyrosine.—In contrast to lysine, the α -hydroxy analogue of phenylalanine, besides being able to replace the latter amino acid in the diet of the growing rat (65), is directly convertible into tyrosine, presumably through phenylalanine, according to experiments reported by Moss (66). In these experiments, deuterio-*dl*- β -phenyllactic acid was added to the casein-containing diet of adult rats over a period of ten days. The deuterium content of the tyrosine obtained from the tissue proteins of these animals was such as to establish its synthesis from the phenyllactic acid fed. Comparison with pre-

viously reported experiments concerned with the conversion of phenylalanine to tyrosine, suggest that phenyllactic acid is only about half as effective as phenylalanine as a precursor of tyrosine. According to Zorn and others (67, 68), the oxidative catabolism of tyrosine in liver and kidney involves neither oxidative deamination nor transamination, the latter because tyrosine, in contrast to alanine, does not donate an amino group to α -ketoglutaric acid. However, in the latter experiment the presence of glutamic acid was tested by isolation as the hydrochloride, so that the possibility of a slow transamination was not excluded.

A continuation of their studies of the relationship of ascorbic acid to the metabolism of tyrosine and phenylalanine has been reported by Sealock and others (69). In the scorbutic guinea pig, the feeding of extra phenylalanine or of phenylpyruvic acid resulted in the excretion of tyrosine metabolites, mainly *p*-hydroxyphenylpyruvic acid, as well as homogentisic and phenylpyruvic acids. Administration of ascorbic acid promptly prevented the excretion of all of these metabolites. The fact that ascorbic acid did not appreciably inhibit the excretion of metabolic intermediates when *p*-hydroxyphenylpyruvic acid was fed to scorbutic guinea pigs suggests the point of attack of the vitamin. The premature infant receiving a fairly high protein, low vitamin C, diet (cow's milk) exhibits a spontaneous defect in the metabolism of the aromatic amino acids similar to that shown by the scorbutic guinea pig, and the condition is accentuated by the feeding of supplements of these amino acids (70). The administration of *L*-ascorbic acid completely eradicates the defect (71) without necessarily raising the ascorbic acid content of the blood plasma. Although these infants exhibit no clinical signs of scurvy, they are doubtlessly in an incipient scorbutic condition. The full-term infant, probably with larger stores of ascorbic acid, does not show the defect spontaneously, but only upon supplementation of his diet with either of the aromatic amino acids. Again the defect is corrected by the administration of ascorbic acid.

According to Bowman (72) the activity of the phenolic group of tyrosine, which is ordinarily only slowly reducing, may be greatly enhanced by the addition of a phosphate buffer, such as might exist in the cells of the body. This finding would appear to strengthen the belief that there is a fundamental relationship between the phenolic hydroxyl of tyrosine and the physiological properties of pepsin, insulin, and the chorionic, gonadotropic and lactogenic hormones, of which it is an integral part. The phenolic group of tyrosine has also been

credited with a dominant role in determining the immunological character of proteins.

Histidine.—Injection of natural *l*-histidine into guinea pigs causes a six- or sevenfold increase in the histamine content of the lung (73). While *d*-histidine will show the same result, the effect is much slower. From these facts, Edlbacher and co-workers conclude that the histidine decarboxylase of the kidneys can gradually be reoriented to decarboxylate the unnatural isomer. There is a considerable literature on disturbances in the metabolism of histidine during pregnancy. In pregnant as compared with nonpregnant women the histaminase activity of the blood is greatly increased (74); it reaches a peak at seven months and then decreases. Histidinuria is a characteristic of normal human pregnancy (75), but is considerably diminished or entirely absent in patients with serious symptoms of preeclamptic toxemia. In the latter condition histamine appears in the urine (76) and is assumed by Kapellar-Adler (77) to be a causative factor in the toxemia of pregnancy. Langley (78) has made a statistical study of the occurrence of histidine in 104 nonpregnancy urines and 107 pregnancy urines. While the creatinine to histidine ratio averaged 3.28 times as high in pregnancy as in nonpregnancy urine, a study of the distributions of the ratio in the two series of urines showed that the ratio cannot be considered a reliable criterion of pregnancy, as about 16 per cent of the urines could not be definitely classified. The estimation of the ratio may be recommended, however, as a valuable diagnostic aid. After injection of histidine into patients with various skin diseases, or introduction into the skin by galvanic current, followed by local exposure to ultraviolet irradiation "to produce histamine *in vivo*," Järnecke (79) claims to have secured satisfactory therapeutic results. Control injections of leucine, cystine, and tyrosine followed by irradiation were ineffective, though an inflammatory or pallor change after tyrosine suggested to the author a production *in vivo* of tyramine or of epinephrine, respectively.

Cystine and methionine.—The synthesis of cystine by the growing rat has been demonstrated conclusively by Rose & Wood (80), using synthetic amino acid mixtures devoid of cystine; this confirms the less exacting experiments of Beach & White (81). The rate of synthesis averaged 3.55 mgs. per gm. gain in body weight; neither the cystine increment nor the rate of gain was modified by the inclusions of cystine, as well as methionine, in the amino acid mixture. These experiments, and others of similar nature, prove only that the sulfur of methionine

is used in the synthesis of cystine. Employing a new method in the study of sulfur metabolism (82), involving the analysis of the ultrafiltrates of blood plasma, Brown & Lewis (83) have shown that the cystine content of the plasma ultrafiltrate increases after the administration, either orally or subcutaneously, of *dl*-methionine. This is in further confirmation of the belief that cystine may be synthesized from methionine in the animal body. The results indicate also a probably slower metabolism of methionine than of cystine, since the rate of increase of sulfate sulfur in the plasma ultrafiltrates was slower after the administration of methionine than of cystine.

The method of conversion of methionine into cystine is still unknown. Blood & Lewis (84) report studies on the metabolism of S-carboxymethylcysteine, the results of which, as the study of a model reaction, offer no support to the hypothesis of Brand and others (85) that the conversion involves the formation of a mixed disulfide which on cleavage yields cysteine and homocysteine.

The fate of the various sulfur-containing amino acids in cystinuria has been studied by Lough *et al.* (86). Cystine and homocysteine, but not cysteine, yielded extra cystine in the urine. Methionine yielded cystine only when the diet fed was moderately low in protein (1.2 gm. per kgm.). The cystinuria could be eliminated by the feeding of 12 gms. of S-carboxymethylcysteine over a period of three days.

The main product in the first stage of the metabolism of *dl*-methionine by liver and kidney slices is the corresponding α -keto acid (87). Sulfate is produced to a slight extent, but no -S-S-, sulfhydryl, or thiolactone groups could be detected by the nitroprusside reaction. Neither homocystine nor homocysteine is deaminized by tissue slices.

Having previously shown (88) that *dl*-methionine sulfoxide can replace *dl*-methionine in the diet of the growing rat, Bennett (89) extended her studies to other partially oxidized derivatives. The sulfone derivative proved incapable of substitution, but the methyl sulfonium chloride, after a lapse of six days, promoted growth as rapidly as methionine itself, suggesting the development of an adaptive mechanism for the advantageous utilization of this unusual dietary component. The incomplete reports of these experiments prevent any appraisal of the competency of the supporting data.

Methionine sulfoxide, like methionine itself, promotes the formation of taurocholic acid in bile fistula dogs (90), suggesting, though far from proving, that it may be an intermediate in the oxidation of methionine to taurine. The results confirm previously reported data

on cystine in showing that the more highly the sulfur is oxidized in the compound administered, the more difficult becomes the biological oxidation of its sulfur to sulfate. Du Vigneaud, Wood & Binkley (91) have reported the biological acetylation of *d*-cystine, an experimental finding that bears on the mechanism of the inversion of *d*-amino acids to their antipodes in the animal body.

Womack & Rose (92), in ad libitum feeding experiments on growing rats, show that, although a dietary supplement of cystine exerts no growth-promoting effect in the complete absence of dietary methionine and when it is present in a concentration of 0.6 per cent or more, it may promote growth when the dietary concentration of methionine is less than that amount. The data suggest that approximately one-sixth of the methionine "requirement" of the rat can be satisfied by cystine, by sparing methionine that otherwise would be used to form tissue cystine.

Amino acid requirements.—Although the growing chick differs from the growing rat in possessing a definite requirement for glycine (93), a much more intense requirement for arginine (94), and an apparent inability to convert ornithine into arginine (95 as contrasted with 96), it is similar to the rat in its need for methionine (97), in its inability to convert cystine to methionine and in its ability to convert homocystine to methionine only by the aid of labile methyl (choline). According to Hegsted and colleagues (98), the arginine and glycine requirements of the chick are much more intense for a rapid feathering breed (Leghorn) than for a slowly feathering breed (Plymouth Rock), the distinction evidently being related to the fact that feathers contain as high as 6.0 per cent of arginine and 9.5 per cent of glycine (99). Arginine and glycine also prevent a typical paralysis in chickens, characterized by a high-stepping stilted gait with the hocks thrust forward and the toes extended. The organic lesion, however, is in the spinal cord, not in the muscles. The chick also possesses a definite requirement of tryptophane (100), although the required dietary concentration (0.5 per cent of the diet) suggested by experiments that cannot be considered well adapted to quantitative work is 2.5 times the necessary dietary concentration tentatively given by Rose (65) for the rat.

The cystine requirement seems to be definitely increased in lactation, both for the rat (101) and, much less certainly, for the human (102). The requirement of cystine or methionine is also increased by the ingestion of organic substances which require these amino acids for their detoxication, such as iodoacetic acid (103) or *p*-di-

methylaminoazobenzene (104). The addition of iodoacetic acid to a low-protein diet induces in growing rats a statistically significant lowering of the total sulphhydryl content of the carcass as contrasted with control rats on the basal diet alone (105).

The problem of the dietary requirement of the ruminant for amino acids involves the synthetic activity of the microorganisms of the paunch. The functions of this organ as a buffer between the animal and its nutritive supply has been amply shown with reference to the vitamins of the B-complex. That it functions as a protein factory also was first suggested by Weiske (106) in 1879 in Germany and after many decades of ineffective experimental travail in that country its demonstration has been forthcoming. In America, confirmation was soon secured, and during the current year the quantitative aspects of these synthetic powers with reference to the utilization of urea as a protein substitute in the ruminant, as contrasted with the nonruminant (107), has been studied (108, 109). At the University of Wisconsin, which had already done effective work in this field, Wegner and others (110) have studied the chemical changes that occur in the paunch contents of cattle maintained on rations containing and not containing urea, and the effect of the protein content of the rations on the course of protein synthesis (111). The interpretation of such changes in an organ from which a continuous flow of material, not necessarily representative of the original mixture, is emerging is admittedly difficult. The practical application of this knowledge to the preparation of ammoniated feed for cattle and sheep has been indicated by Millar (112) and the significance of it to the war problems of England has been discussed by Benesch (113).

CREATINE METABOLISM AND LABILE METHYL GROUPS

Creatine synthesis.—The perplexing problems of creatine metabolism, particularly the problem of the precursors of body creatine, have been very largely solved during the past year. Pre-eminent importance attaches to the work with compounds marked with the nitrogen isotope, because of the direct evidence thus afforded. Bloch, Schoenheimer & Rittenberg (13) have shown that in the adult rat the daily creatinine elimination in the urine corresponds to approximately 2 per cent of the total body creatine. These losses are approximately equal to the daily synthesis, as measured by the decreasing proportion of isotopic nitrogen in the urinary creatinine excreted and hence in the body creatine from which it was formed (114). This close ap-

proximation, together with the fact that the urinary urea excreted by rats given isotopic creatine contains no significant concentration of marked nitrogen, and the urinary ammonia practically none, "excludes the occurrence of any major metabolic pathway other than that of creatinine formation and synthesis." The irreversibility of the reaction $\text{creatinine} \rightarrow \text{creatinine}$ in the rat was clearly indicated in an earlier experiment from the same laboratory (114). In an extensive study of the biological precursors of creatine, Bloch & Schoenheimer (14) secured clear evidence, in conjunction with recent observations from other laboratories, indicating that the methyl group of creatine is derived from methionine, and that at least the main pathway for the biological synthesis of body creatine involves two reactions: glycine reacts with the amidine group of arginine to form guanidoacetic acid, and the latter is methylated to form creatine by shift of the methyl group from methionine. The slow rate of creatine formation in the body seems to be mainly determined by the slow rate of formation of guanidoacetic acid. Other amino acids are not effective creatine precursors. Sarcosine is not an intermediate until it is demethylated in the body to form glycine. The biological demethylation of sarcosine has been independently proven (115) in experiments with broken cell preparations of liver. Contrary to the theory of Beard (116), urea does not contribute its nitrogen to body creatine (14). The formation of guanidoacetic acid (glycocylamine) in animal tissues (117) and in man (118) has been clearly shown by Borsook and co-workers, using an improved analytical method for guanidoacetic acid (119). Evidence that the conversion of guanidoacetic acid to creatine occurs in the liver has been reported by Bodansky and others (120), who also found creatine in the intestinal contents and feces of rats, even during a period of fasting preceded by the feeding of a creatine-free diet. In rats fed guanidoacetic acid, and in nephrectomized rats, the creatine in intestinal contents and in feces was increased. These results indicate that creatine is excreted by way of the alimentary tract as well as the kidney. If this finding can be confirmed, the calculation of Bloch & Schoenheimer (13) on the rate of synthesis and catabolism of body creatine will need revision. The transfer of methyl groups from methionine to choline and creatine, and from choline to creatine has been observed by du Vigneaud and associates (121); the experiments were performed on rats which were fed appropriate compounds containing deuterium in the methyl groups.

The mechanism of creatine formation in the chicken seems to be

essentially the same as in the mammal, glycine and arginine being important precursors (98, 122), with guanidoacetic acid a potent creatine former. The process of creatine formation in the chick seems to be more closely geared to body growth than in the rat, since with the basal diet used, those supplements that promoted creatine formation also promoted growth. The creatine content of the muscle of the chick is also much less stable than in the rat, while the reaction creatine \rightleftharpoons creatinine appears to be reversible, since dietary creatinine is potent in raising a subnormal content of muscle creatine (122). The last two facts may explain the variable endogenous nitrogen catabolism of the chicken (123), which precludes the accurate determination of the biological value of protein in individual birds.

Labile methyl groups.—The methyl group in neither creatine, creatinine, nor sarcosine is sufficiently labile in the rat to promote the synthesis of methionine from homocystine (124), as does the methyl group of choline and of betaine (125, and previous investigators). The need for choline in the nutrition of the chick is indicated by the experiments of Hegsted and associates (126), more being required for the promotion of maximum growth than for the prevention of perosis. Jukes (127), however, claims that choline is not essential for the prevention of perosis in chicks unless gelatin or creatine is added to the choline-free diet. The rat can evidently synthesize choline (128), if not always at a rate sufficient to cover the need for labile methyl groups. The mechanism of this synthesis in the rat has been explored by Stettin (129), using as dietary supplements ethanolamine, choline, glycine, and ammonia that had been labeled with N^{15} . The results were interpreted to indicate that choline may be synthesized from glycine through ethanolamine, the methylation of which by betaine (or other donor of labile methyl) produces choline. According to Griffith (130), methionine may also supply methyl groups for the synthesis of choline. The quantitative relationships between dietary methionine and cystine, either free or combined in proteins, and dietary choline in the prevention of hemorrhagic degeneration of the kidneys, are revealed in this study. Only methionine and cysteine in a series of ten amino acids tested by Perlman, Stillman & Chaikoff (131), were effective in stimulation of the phospholipid activity of the liver. The lipotropic activity of various derivatives of methionine and cystine has been investigated by Singal & Eckstein (132), who showed among other things that cysteine loses its characteristic effect in promoting the production of fatty livers when it is alkylated with

the methyl, ethyl, propyl, or isopropyl radical, and behaves more like methionine in this respect.

The role of glycine in creatine formation has evidently sustained interest in the possibility, originally suggested by experimental observations reported by Boothby (133), that the ingestion of gelatin, a protein rich in glycine, or glycine itself, would increase muscular endurance and strength. However, negative results with gelatin have been reported from two different laboratories (134, 135). The positive results of Chaikelis (136), indicating statistically significant increases that averaged 17 to 23 per cent in the muscular strength of forty human subjects who received 6 gm. of glycine daily over a period of ten weeks, must be weighed against the negative results of Horvath, Knehr & Dill (137) using similar doses of glycine but with fewer subjects.

SOME PHYSIOLOGICAL EFFECTS OF AMINO ACIDS AND PROTEINS

The specific dynamic effect of histidine, injected intravenously into dogs, was definite and prolonged according to Eaton & Doty (138). The heating effect of a series of six amino acids, over and above that of an adequate maintenance diet, was studied by Kriss (139), using rats as experimental subjects. From the fact that the heat increments were closely correlated with the metabolizable energy contents of the acids, the conclusion (not inescapable) was drawn that the dynamic effects of amino acids are by-products of intermediary chemical reactions. Methionine and cystine have been found (140) to protect protein-depleted dogs against chloroform liver injury. Acute renal hypertension has been produced (141) by the injection of dopa- (*L*-dihydroxyphenylalanine) into the partially or completely ischemic kidney of the cat. A high-protein diet is not fundamentally involved in hypertension, with or without renal damage (142), while proteins from different food sources are differentially involved in experimental atherosclerosis in rabbits (143.)

Investigations on the protective action of proteins against the nutritional cataract induced by excessive galactose feeding indicate that this action does not parallel their value in growth promotion (144), and that it is associated with the diamino and dicarboxylic acid fraction of the enzymic hydrolysates (145). There is considerable evidence that the selenium in toxic wheat and other seleniferous plants has replaced sulfur in some sulfur-containing amino acid (146, 147), and that the known protective action of protein against the toxicity induced

by the consumption of seleniferous grains resides in the sulfur-containing amino acids, particularly methionine (148). The effects of dietary protein (149, 150) and of methionine (151) on the toxicity and therapeutic action of sulfanilamide have been revealed.

The functions of dietary proteins and amino acids are so intimately related to the structure and the life of every cell in the animal body that a protein deficiency in the diet does not induce pathognomonic symptoms in one or another tissue, as does a vitamin deficiency; the functions of all tissues fail together. The only internal symptom of general occurrence is the slow production of a hypoproteinemia, which is associated with a depletion of reserve protein stores (*vide supra*). Such depletion can be experimentally induced by plasmapheresis for the purpose of studying plasma protein regeneration. The stimulus to regeneration increases the lower the hypoproteinemia (152), just as the stimulus to hemoglobin regeneration increases with the severity of the anemia (153). Support for the designation of cystine as a key amino acid in this regeneration (152) is hard to find in the evidence presented, while evidence to the same effect in earlier publications involves the possible supplementing effect of cystine on a cystine-poor basal diet.

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THE CHEMISTRY OF THE HORMONES

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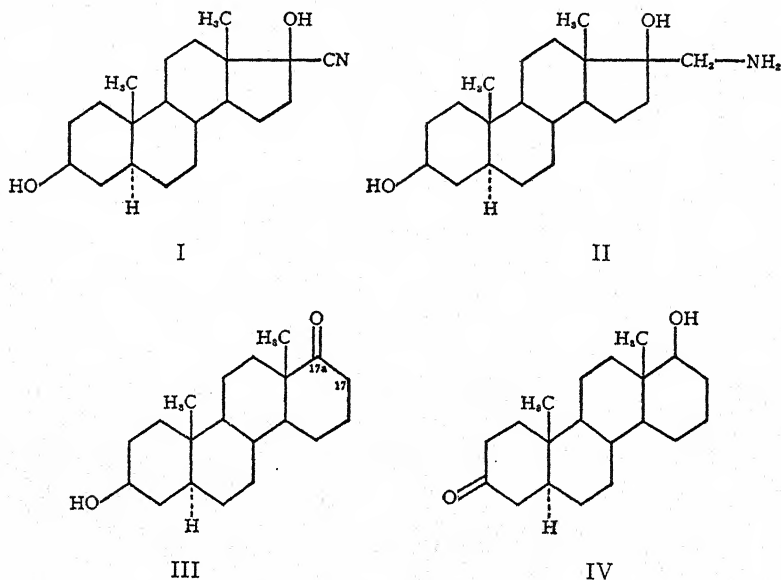
During the period under review steady progress has been made in the several fields of hormone chemistry. As is to be expected from the nature of the work, progress has been more obvious in the field of the steroid hormones than in that of the protein and protein-like hormones. In the latter field interesting preliminary reports have appeared on the isolation of a protein from beef posterior pituitary gland having oxytocic, pressor, and antidiuretic activity (1), and on the separation of the posterior pituitary principles by such "mild" methods as chromatographic adsorption (2). However, the limited number of detailed chemical studies on the protein hormones which have appeared during the past year has prompted the writers to limit this review to those hormones having a steroid nature.

A major share of the total effort has been directed to a continued expansion of the study of the relationship of structure to physiological action. Since the results bear closely on the general problem of the intermediary metabolism of the steroid hormones and their relationship to the urinary steroids the results of chemical studies in these related fields are included. Preparative work on compounds of the testosterone, progesterone, and corticosterone type have been confined to interconversions of the natural steroids. In the estrogen group, Bachman and his co-workers prepared a number of new compounds related to equilenin by methods similar to those which they elaborated some time ago for the total synthesis of *d*-equilenin, itself. Other studies on the total synthesis of steroid hormones are not reviewed since the results achieved beyond those summarized by Springall (3) are not as yet of immediate interest in hormone chemistry. Although it has become increasingly difficult to discuss the steroid hormones in physiological groups such as estrogens, androgens, et cetera, this somewhat artificial division is used for convenience of reference.

ANDROGENS

The size of ring D in the androgens is apparently not highly specific for physiological activity. On the other hand the activity is markedly reduced by the introduction of oxygen at C₈ or C₁₈ or by the

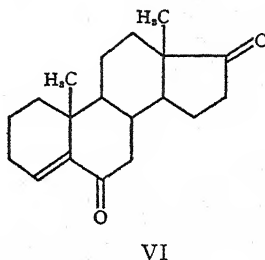
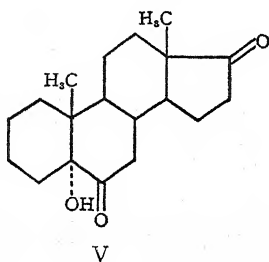
shifting of the double bond to the 1,2 position. Goldberg & Monnier (4) prepared D-homo-dihydrotestosterone (IV)¹ by the usual methods from D-homo-androstane-3(β)-ol-17a-one (III). The latter compound and its 3 epimer were prepared (5) from the corresponding 17-keto compounds through the cyanhydrin (I). The amine (II) obtained on catalytic reduction rearranged on deaminizing with nitrous acid to the D-homo derivative III. The androgenic activity of D-homo-dihydrotestosterone is of the same order as that of dihydrotestosterone and testosterone itself by both the capon and the rat methods.



Moving the oxygen from C₃ to C₆ in 4-androstene-3,17-dione reduces the androgenic activity by about 60 per cent (6). 4-Androstene-6,17-dione was prepared from dehydroisoandrosterone. The 3-benzoate was converted to the 5,6-oxide which was hydrolyzed to the 5,6-diol (*trans*). Following oxidation to the 6-keto derivative the secondary hydroxyl group on C₃ was removed with tosyl chloride in pyridine. The 3,4 double bond was reduced catalytically to yield androstane-5-ol-6,17-dione (V) which was readily dehydrated with acetic anhy-

¹ For the nomenclature of the ring homologues of the steroids see Ruzicka & Meldahl (154).

dride and heat to 4-androstene-6,17-dione (VI). The compound is inactive in the Allen-Doisy test.



The 1,2 bond isomer of testosterone was prepared by Butenandt & Dannenberg (7). The compound 1-androstene-17-ol-3-one was obtained by splitting out hydrobromic acid from 2-bromoandrostane-17-ol-3-one with collidine. The compound described by these authors earlier as 1-androstene-17-ol-3-one (8, 9) is a rearrangement product of unknown constitution and was obtained by the action of potassium acetate in glacial acetic acid on 2-bromoandrostane-17-ol-3-one. These rearrangement products are referred to as the hetero series. The androgenic activity of 1-androstene-17-ol-3-one is comparable to that of 4-androstene-3,17-dione. It is without estrogenic action in contrast to the earlier reported hetero compound which had appreciable estrogenic activity.

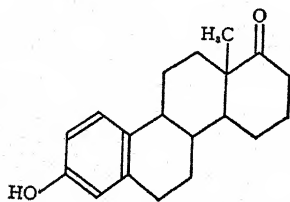
The 6(α)-acetoxy group reduces the androgenic activity of 4-androstene-3,17-dione (capon method) to about one fifth that of androsterone. The compound was prepared (10) by oxidation and dehydration of the 6-acetate of androstane-3(β),5,6(*trans*)-triol-17-one obtained on acetolysis of androstane-3(β)-ol-17-one-(5,6)(α)-oxide.

The difficulty of obtaining 5-androstene-3,16,17-triol has made 16-hydroxytestosterone (11) relatively inaccessible. The triol has now been obtained in good yield by the reduction of 16-isonitroso-isodehydroandrosterone with zinc and acetic acid and reduction of the resulting mixture of 16,17-ketols using Raney nickel catalyst (12). The 16-isonitroso group was introduced by the methods employed by Litvan & Robinson for the preparation of 16-isonitroso-O-methyl estrone (13). 16-Ketotestosterone was prepared (14) for a study of its possible cortin activity since it possesses an α -ketol group in ring D. It is inactive in this respect and will probably be found inactive androgenically since 16-hydroxytestosterone is inactive in both the capon

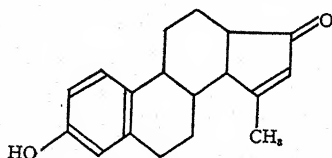
and the castrate rat (11). The 16-keto derivative was prepared from the 3-enol ethyl ether of 4-androstene-3,17-dione (15). The 16-benzal derivative was reduced to the 17-alcohol. The 3-enol ether was converted to the 3-ketone and then after acetylating the 17-hydroxyl group the semi-cyclic double bond was opened with osmium tetroxide and the 16,16 α -diol oxidized with periodic acid to 16-ketotestosterone acetate.

ESTROGENS

The effect of enlargement of ring D on the activity of estrone was established by Goldberg & Studer (16). They prepared D-homo-estrone (VII) by methods similar to those which were used to prepare D-homo-androsterone and D-homo-iso-androsterone (5). Enlarging ring D to six members reduces the activity thirty times as measured by the Allen-Doisy test.



VII



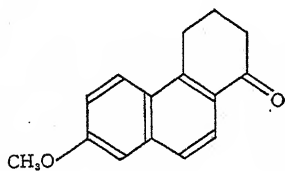
VIII

The size of the fourth ring is apparently of less significance in determining the estrogenic effect than the relative positions of the methyl and carbonyl groups on the ring since a compound of the type VIII² has less than one one-hundredth the activity of estrone (17).

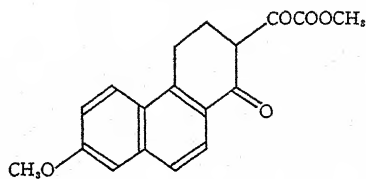
The total synthesis of *d*-equilenin (natural compound) and its three space isomers has ushered in a new approach to the study of the relationship of structure to physiological action in the estrogen series. 7-Methoxy-1-keto-1,2,3,4-tetrahydrophenanthrene (IX) was condensed with methyl oxalate to yield the glyoxalate X which lost carbon monoxide on heating. The resulting keto ester was converted to its sodio derivative and reacted with methyl iodide to yield the 2-methyl derivative XI which underwent the Reformatsky reaction with methyl bromoacetate to give the dicarboxylic acid ester XII.

² The position of the methyl and α - β unsaturated ketone group on the fourth ring is not certain.

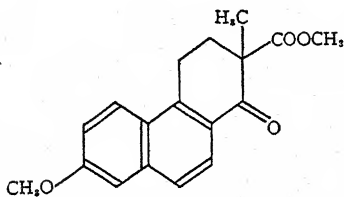
This intermediate was dehydrated by replacing the hydroxyl group with chlorine and splitting out hydrochloric acid, the acid XIII and its geometric isomer being obtained. On reduction (sodium amalgam and water) the acetic acid derivative XIV was obtained as two racemic mixtures. In each the acetic acid sidechain was lengthened by one methylene group by the method of Arndt & Eistert and the dimethyl esters of the racemic 7-methoxy-2-methyl-2-carboxy-



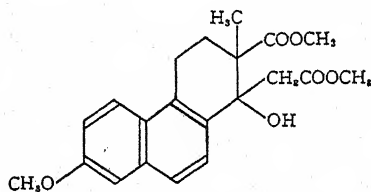
IX



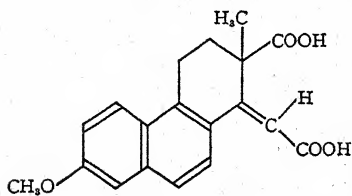
X



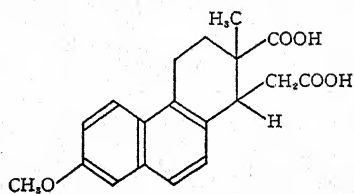
XI



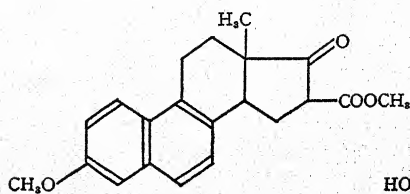
XII



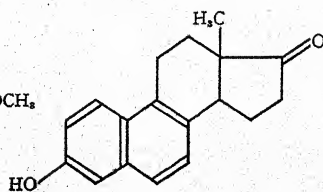
XIII



XIV



XV



XVI

1,2,3,4-tetrahydrophenanthrene-1-propionic acids were cyclized with sodium methoxide in benzene solution to the *racemic* 16-carbomethoxy methyl ethers (XV). On heating with hydrochloric and acetic acid the ether linkage was split, the methyl ester group hydrolyzed and the acid decarboxylated to yield *dl*-equilenin and *dl*-isoequilenin (XVI). Both racemic mixtures were resolved and the synthetic *d*-equilenin found to be identical with the natural product. Bachman *et al.* have extended this work to the preparation of a number of related compounds using similar procedures. They are enumerated in Table I.

TABLE I

Compound	Estrogenic Activity $\mu\text{g.}$	Reference
<i>d</i> -Equilenin	30	(19)
<i>l</i> -Equilenin	400	(19)
<i>dl</i> -Equilenin	60	(20)
<i>d</i> -Isoequilenin	> 500	(19)
<i>l</i> -Isoequilenin	> 500	(19)
α - <i>dl</i> -17-Equilenone*	> 500	(19)
β - <i>dl</i> -17-Equilenone	> 500	(19)
α - <i>dl</i> -6-Hydroxy-17-equilenone	> 500	(21)
β - <i>dl</i> -6-Hydroxy-17-equilenone	> 500	(21)
α - <i>dl</i> -6-Hydroxy-19-methyl-17-equilenone	>1,000	(21)
β - <i>dl</i> -6-Hydroxy-19-methyl-17-equilenone	>1,000	(21)
α - <i>dl</i> -6-Hydroxy-D-homo-17a-equilenone	>1,000	(21)
β - <i>dl</i> -6-Hydroxy-D-homo-17a-equilenone	>1,000	(21)
α - <i>dl</i> -3-Hydroxy-19-methyl-17-equilenone	>1,000	(22, 20)
β - <i>dl</i> -3-Hydroxy-19-methyl-17-equilenone	100†	(22, 20)
α - <i>dl</i> -3-Hydroxy-19-ethyl-17-equilenone	250	(20)
β - <i>dl</i> -3-Hydroxy-19-ethyl-17-equilenone	25	(20)
β - <i>dl</i> -3-Hydroxy-19-propyl-17-equilenone	>1,000	(20)
<i>dl</i> -16-Methylequilenin	> 500	(20)
<i>dl</i> -16-Methylisoequilenin	>1,000	(20)
<i>dl</i> -D-Homoequilenin	>1,000	(20)
<i>dl</i> -D-Homoisoequilenin	100	(20)

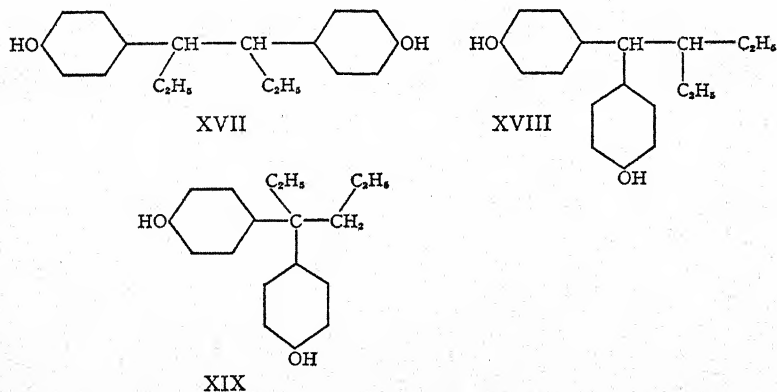
* This system of nomenclature is based on the hydrocarbon, equilenane (see reference 23). The prefixes α and β are used instead of *cis* and *trans* since the spatial orientation is not known.

† The relatively high order of activity is interpreted as indicative that this racemate has the same configuration as *dl*-equilenin.

Particularly striking is the fact that the replacement of the angular methyl group in *dl*-equilenin with the propyl group has little effect on the estrogenic potency. On the other hand *x*-norequilenin acetate (no

angular alkyl) is reported to be a very weak estrogen (positive test with a 10 mg. dose) (18). However, in the latter compound the configuration relative to natural equilenin is unknown around C_{13} and C_{14} . It would appear that enlarging the fourth ring to six members in equilenin (*dl*-D-homo-equilenin) has much less effect on estrogenic activity than it does in estrone (see D-homo-estrone above).

Stilbestrol and related compounds.—Convenient methods for the preparation of hexestrol [α : δ -di-(4-hydroxyphenyl)hexane] (XVII) in satisfactory yield from readily available starting materials have been described. Docken & Spielman (24) converted anethole to its hydrobromide and coupled it by means of magnesium in ether to give the dimethyl ether of hexestrol. Bernstein & Wallis (25) started with *p*-hydroxypropiophenone preparing the α -bromide of anethole in three steps. They coupled the bromide with sodium in ether. Demethylation to the free phenol is smoothly effected with either alcoholic potassium hydroxide (26, 24) or hydriodic acid (25, 27). A number of oil-soluble esters of hexestrol such as the propionate, butyrate, benzoate, and caproate have been prepared and are reported to be less potent than hexestrol whereas the disuccinate in aqueous solution is considered as active as hexestrol (27). Campbell (28) found two position isomers (XVIII and XIX) relatively inactive as compared to hexestrol



(XVII) although they were by no means inactive. 3,3'-Nuclear methyl groups in XIX enhanced its estrogenic effect four or five times. The chain length of 6 or 7 carbon atoms was found to be the optimum length in homologous series of 4:4'-dihydroxydiphenylmethane derivatives (28).

In the homologous series of dimethyl to dipropyl ethers of diethylstilbestrol the oral estrogenic activity decreases markedly (29). The importance of the position of the phenolic groups in diethylstilbestrol was examined. 3-, 3:3'-, and 3:4'-dihydroxy- α : β -diethylstilbenes were prepared and found to be very much less active than the 4:4'-dihydroxy derivative (diethylstilbestrol) (30, 31). α,α -Di-*p*-methoxyphenyl- β -phenylbromoethylene has been found to be a much more powerful estrogenic agent than triphenylchloroethylene (32, 33, 34). The demonstration (35) that 4'',4'''-dihydroxyquaterphenyl is devoid of estrogenic action is of interest in connection with the appreciable estrogenic effect of 4:4'-dihydroxydiphenyl (36).

Benzoylcarbinol and 3'-(ω -hydroxyaceto)-4-hydroxy- α : β -diethylstilbene are reported to exhibit slight cortin activity in young adrenalectomized rats (37). Negative results, however, have been reported with hexahydrobenzoyl carbinol, several lower alkyl α -ketols, naphthoyl carbinol (37, 38), and decahydronaphthoyl carbinol (39).

PROGESTERONE

A number of sidechain homologues of progesterone have been prepared and assayed for progestational effect. The results make it clear that the length of the sidechain in progesterone is highly specific. 20-Nor-progesterone (17-formyl-4-androstene-3-one) was found to have slight progestational activity at the high dose level of 10 and 20 mg. It is also slightly androgenic (one international capon unit in 1 mg., local application) (40, 41). The known 4-pregnene-20,21-diol-3-one (42) gave the compound in good yield on periodic acid oxidation. 21-Methyl-progesterone showed progestational activity in 3 to 5 mg. doses while the 21-ethyl derivative was inactive at 40 mg. They were both inactive orally (43). The sidechain was lengthened by reacting 3(β)-acetoxy-5-etiocholenic acid chloride either in a malonic or substituted malonic ester synthesis, or with the appropriate metal alkyl. A homologue of progesterone with the sidechain $-\text{CH}(\text{CH}_3) \cdot \text{CO} \cdot \text{CH}_3$ is entirely inactive at a 30 mg. dose level. This sidechain was built up by the action of 3(β)-acetoxy-5-norcholelenic acid chloride with methyl zinc iodide (44). The progesterone homologue having an acetonyl ($-\text{CH}_2 \cdot \text{CO} \cdot \text{CH}_3$) sidechain instead of acetyl ($-\text{CO} \cdot \text{CH}_3$) is inactive at 20 mg. (45). The acetonyl sidechain was obtained by reacting methyl zinc iodide with 3(β)-acetoxy-5,17-pregnadienic acid-21 and reducing the 17,20 double bond selectively with Raney nickel catalyst. It is not certain that the sidechain

in the acetonyl derivative has the same configuration as in progesterone.

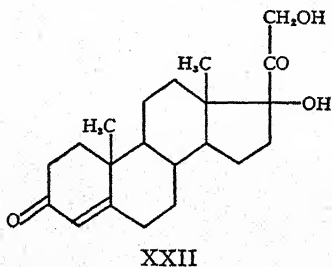
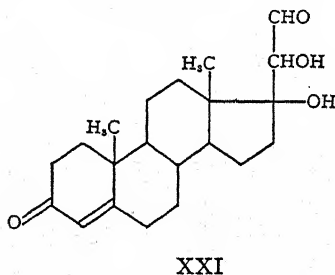
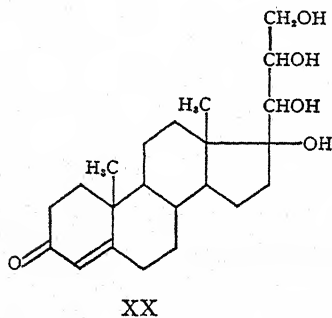
11-Hydroxyprogesterone is readily dehydrated on warming in a mixture of hydrochloric and acetic acids to 11-dehydropregesterone (46, 47). The position of the double bond at 11,12 is not proved but its mode of preparation is in favor of that formulation. The 11-hydroxyprogesterone was prepared by converting corticosterone to its 21-iodide and removing the iodine with zinc and acetic acid (48). Whereas 11-hydroxyprogesterone is less than one sixth as active as progesterone, 11-dehydropregesterone is at least one half as active. 6-Dehydropregesterone has appreciable progestational activity (49) while 17(β)-hydroxyprogesterone (50) and 16-dehydropregesterone (51) are entirely inactive. Perhaps the slight progestational effect of the 11-hydroxy- and the 6(α)-acetoxyprogesterone (52, 53) is due to *in vivo* dehydration to the corresponding dehydropregesterones.

A new starting material for the preparation of progesterone as well as testosterone and desoxycorticosterone has been found in diosgenin (54 to 58). The structure of rings A and B in this sapogenin is identical with that of the corresponding rings in cholesterol. Acetic anhydride and heat convert diosgenin to the diacetate of the pseudogenin which yields the acetate of 5,16-pregnadiene-3(β)-ol-20-one on oxidation with chromic acid under mild conditions. The 16,17-double bond is selectively reduced with hydrogen over palladium catalyst or zinc and acetic acid. The resulting acetate of 5-pregnene-3(β)-ol-20-one was converted to progesterone by customary methods. Oxidative removal of the acetyl sidechain or conversion to the α -ketol sidechain of desoxycorticosterone was effected with persulfuric acid in acetic acid.

ADRENAL STEROIDS

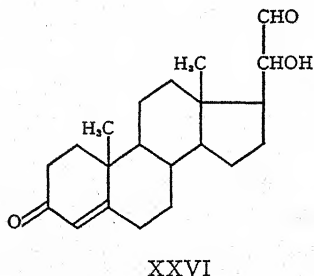
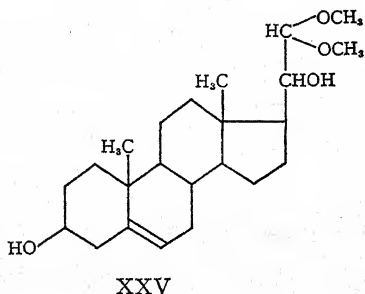
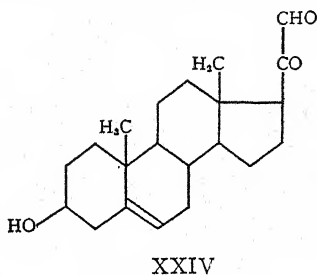
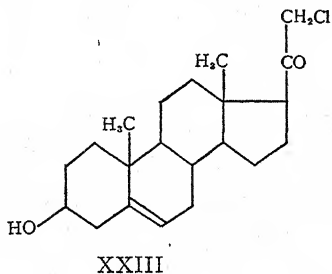
Desoxycorticosterone and related ketols and hydroxy aldehydes.—17-Hydroxydesoxycorticosterone (substance S) (XXII) has been prepared by partial synthetic methods making the compound available for physiological study (59). The quantity isolated from the gland by Reichstein in 1938 was sufficient only for structural analysis (60). Although no observations are available on the cortin activity of this compound it can be expected to have some activity in maintaining adrenalectomized animals. The effect of this hormone on the sodium and chloride excretion will be of particular interest since Thorn, Engel & Lewis (61) have recently shown that the cortex hormones bearing

a 17-hydroxyl group (17-hydroxycorticosterone and 17-hydroxydehydrocorticosterone) induce a negative salt balance in dogs as compared to those cortex hormones without a 17-hydroxyl group (corticosterone, dehydrocorticosterone, desoxycorticosterone) which induce a positive salt balance. 17-Hydroxydesoxycorticosterone is inactive in the Ingle work test and is nondiabetogenic in the partially depancrea-
 tized rat (62, 63). The compound was obtained by the rearrangement of 4-pregnene-17,20-diol-3-one-21-al (XXI) in boiling pyridine, a method first used for the rearrangement of glyceraldehyde to dihydroxy acetone by Fischer, Taube & Baer (64). This aldehyde as well as 4-pregnene-20-ol-3-one-21-al (XXVI) was prepared in the hope of throwing light on the possibility that α -hydroxy aldehydes may account for some of the activity of cortex extracts and particularly of the amorphous fraction (65, 66). The 17-hydroxy aldehyde (XXI) was prepared from the trihydroxypropyl testosterone (XX) of Bute-



nant & Peters (67). The β - γ acetone addition product was acetylated, acetone split off and the product oxidized to the 20-acetate of XXI with periodic acid. Hydrolysis with alcoholic potassium bicar-

bonate gave the free compound (XXI). Trihydroxypropyltestosterone has also been converted directly to the aldehyde (XXI) by stoichiometric oxidation with periodic acid (68). Since 17-hydroxydesoxycorticosterone was demonstrated earlier to be of the 17 β -series³ (69) it follows that the testosterone derivative XX has the β configuration.

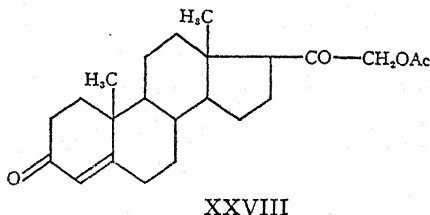
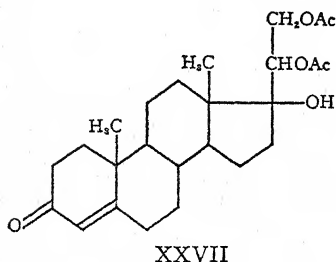


4-Pregnene-20-ol-3-one-21-al (XXVI) was prepared from 21-chlor-5-pregnene-3(β)-ol-20-one (XXIII). The α -keto aldehyde XXIV was obtained in good yield (70) from the chlor-derivative XXIII (71) by the method of Kröhnke & Börner (72). A Meerwein-Ponndorff reduction of the dimethyl acetal of XXIV gave a diol XXV. Oxidation in the 3-position by Oppenauer's method and hydrolysis of the acetal group gave the α -hydroxy aldehyde XXVI. No

³ The terms α and β at C_{17} are used as defined by Reichstein & Gätzi (155), and α and β at C_{20} as defined by Prins & Reichstein (156). It should be noted that the latter terms have not been related to the α and β at C_{20} as used by Marker *et al.* (157) for the 20-pregnans, α being used here to designate the configuration at C_{20} as in the pregnanediol described by Marrian (158). Epimerism at C_8 is designated as suggested by Fieser, "Chemistry of Natural Products Related to Phenanthrene," 2nd ed., pp. 113-17.

bioassay data are available as yet on this compound or its 17-hydroxy analogue.

Assay results on 17-iso-desoxycorticosterone (XXVIII) are in keeping with earlier findings on the relative potencies of progesterone and isoprogesterone, the iso compound being much the less active (73). The triol diacetate XXVII described earlier by Ruzicka &



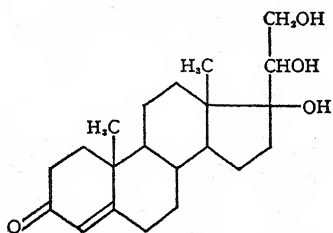
Müller (74) which is of the 17 β -series lost acetic acid on heating with zinc dust and acetic acid to give the 21-acetate of 17-iso-desoxycorticosterone (XXVIII). This readily rearranged to desoxycorticosterone on heating with hydrochloric acid (75, 73).

Ehrenstein (10) prepared the diacetate of 6(α)-hydroxydesoxycorticosterone using methods comparable to those mentioned earlier for the preparation of 6(α)-hydroxyandrostene-4-dione-3,17. The 6(α)-acetoxy group reduces the life maintenance activity of desoxycorticosterone as measured in the adrenalectomized rat by at least fifteen times. Like desoxycorticosterone it is inactive in the Ingle work test and has no diabetogenic effect. A homologue of desoxycorticosterone with the sidechain, $-\text{CH}(\text{CH}_3) \cdot \text{CO} \cdot \text{CH}_2\text{OAc}$ (44) and 6-dehydrocorticosterone (49) were obtained in the course of preparing the analogous progesterone derivatives. Both were inactive in maintaining the life of the adrenalectomized rat. The tetraacetyl- β -glucoside of desoxycorticosterone has been described (76).

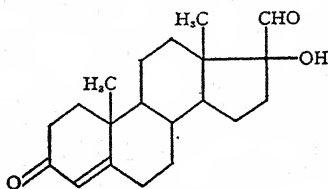
17-Hydroxyprogesterone and related D-homo compounds.—Isolation of 17-hydroxyprogesterone (50, 77) and 4-androstene-3,17-dione (77) has brought the number of steroids isolated from adrenal cortex extracts to a total of twenty-five. It was suggested that the 4-androstene-3,17-dione might arise from the oxidation of 17-hydroxydesoxycorticosterone or related compounds during the process of isolation since only a very small quantity of androstenedione was obtained (77). As might be expected 17-hydroxyprogesterone has no cortin activity.

It is likewise devoid of progesterone activity but exhibits androgenic potency in the castrate rat (50). A new physiologically inactive α - β unsaturated ketone analyzing as $C_{21}H_{28-30}O_4$ and most likely of steroid nature was also isolated from cortex extracts (78).

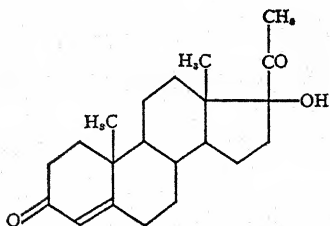
The natural 17-hydroxyprogesterone was prepared by partial synthetic methods using several different routes (79, 80). The best method is that of Prins & Reichstein (80). 4-Pregnene-17(β),20(β),21-triol-3-one (XXIX) (74, 81) was oxidized with periodic acid to the aldehyde XXX which gave 17(β)-hydroxyprogesterone (XXXI) on treatment with diazomethane. This conversion proved the 17(β)-configuration in the natural 17-hydroxyprogesterone.



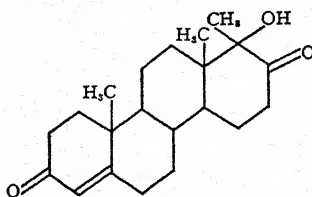
XXIX



XXX



XXXI

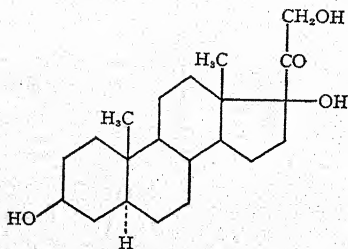


XXXII

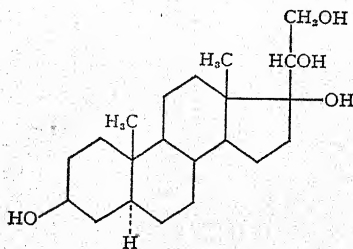
Von Euw & Reichstein (77) observed that 17(β)-hydroxyprogesterone (XXXI) on treatment with heat, strong alkali, or aluminum tertiary butylate rearranged to 17 α -methyl-D-homo-4-androstene-17 α -ol-3,17-dione (XXXII) identical with the compound obtained earlier by Ruzicka & Meldahl (82) in their attempted preparation of 17(α)-hydroxyprogesterone. An attempt to prepare 17(β)-hydroxyprogesterone (XXXI) by oxidation of 5-pregnene-3(β),17(β)-diol-20-one with acetone and aluminum tertiary butylate led to the same rearrangement product (XXXII) (79). The isolation of the rearrange-

ment product was accomplished with the aid of chromatography on aluminum oxide. Stavely (83) found that rearrangement of 17(α)-hydroxy-20-ketones of the pregnene series to the D-homo-androstene series could be effected by simply pouring a benzene solution of the hydroxy ketone through a column of alumina. He demonstrated that 5-pregnene-3(β),17(α)-diol-20-one rearranged to a 17a-methyl-D-homo-5-androstene-3(β),17a-diol-17-one by this means (83). 17(β)-Hydroxyprogesterone, however, was not rearranged on chromatographing over aluminum oxide (77, 80). Oxidation of Stavely's diol with aluminum isopropylate yielded a new D-homo androstene derivative which was shown to be the 17a epimer of Ruzicka & Meldahl's 17a-methyl-D-homo-4-androstene-17a-ol-3,17-dione (XXXII). Stavely's epimer is progestationally active at a 12.5 mg. dose level. The higher melting epimer obtained by Ruzicka & Meldahl (82) from the 17(α) series and by von Euw & Reichstein (77) from the 17(β) series is inactive progestationally at 20 mg. but the 17a-acetate is active at 30 mg. (Clauberg technique). The higher melting isomer has also been reported to be about one eighth as active as desoxycorticosterone in the adrenalectomized dog (84).

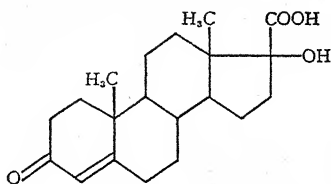
Physiologically inactivated saturated compounds.—As a result of studies from Reichstein's laboratory all of the known adrenal steroids having an hydroxyl group on C₁₇ but having no oxygen on C₁₁ have been directly correlated with respect to the configuration at C₁₇ and all seven have been prepared by partial synthetic methods. The preparation of 17(β)-hydroxyprogesterone and 17-hydroxydesoxycorticosterone (substance S) has already been discussed, the β -configuration of the natural 17-hydroxyprogesterone (XXXI) following from its mode of preparation. The 17 β -configuration of 17-hydroxydesoxycorticosterone (XXII) was demonstrated earlier by its degradation to the known 3-keto-17(β)-hydroxy-4-etiocholenic acid (XXXV) (69).



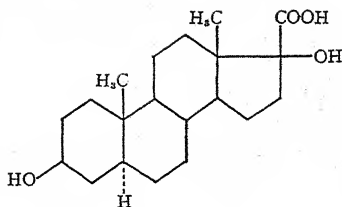
XXXIII



XXXIV

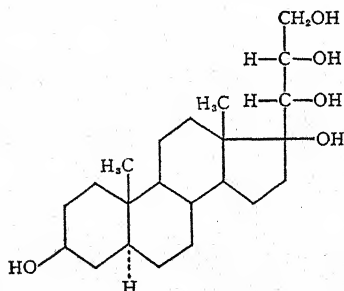


XXXV

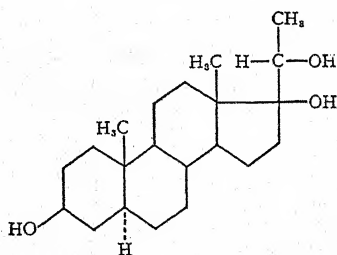


XXXVI

Allopregnane-3(β),17(β),21-triol-20-one (XXXIII) (substance P) on reduction yielded allopregnane-3(β),17(β),20(β),21-tetrol (XXXIV) (substance K) and its C_{20} epimer. The tetrol (XXXIV) yielded 3(β),17(β)-dihydroxy-allo-etiocholanolic acid (XXXVI) on periodic acid oxidation (85, 86). Allopregnane-3(β),17(β),21-triol-20-one (XXXIII) (substance P) was prepared by methods similar to those discussed above for the preparation of 17-hydroxydesoxycorticosterone (substance S) (87). The intermediate allo-homo-(ω)-pregnane-3(β),17(β),20(β),21(β),22-pentol⁴ (XXXVII) (87) was obtained by methods similar to those employed by Butenandt & Peters



XXXVII



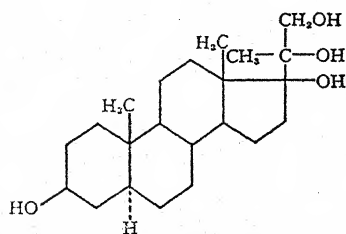
XXXVIII

(67) in their preparation of trihydroxypropyl testosterone (XX). The availability of substance P (XXXIII) makes substance K (XXXIV) readily obtainable by reduction.

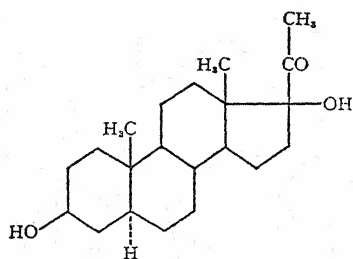
The tetrol XXXIV (substance K) was converted to allopregnane-3(β),17(β),20(β)-triol (substance J) (XXXVIII) and its C_{20}

⁴ The term allo-homo- ω -pregnane was suggested for the parent hydrocarbon of compound (XXXVII) and the arbitrary β designation of the hydroxyl groups on C_{20} and C_{21} is discussed by von Euw & Reichstein [pp. 403-4 (87)].

space isomer, allopregnane-3(β),17(β),20(α)-triol (substance O), by oxidation with periodic acid to 17-formyl-androstane-3(β),17(β)-diol which with methyl magnesium bromide gave a mixture of the two triols J and O (88). Since these two triols had been obtained earlier (89) by the reduction of allopregnane-3(β),17(β)-diol-20-one (substance L), the above conversion of the tetrol (XXXIV) to the two triols is direct evidence of the C_{17} - β configuration in substance L. This evidence is in keeping with that from the partial synthesis of allopregnane-3(β),17(β)-diol-20-one (substance L) from substance P [allopregnane-3(β),17(β),21-triol-20-one] (XXXIII). Treatment of substance P with methyl magnesium bromide yielded a mixture of the C_{20} epimers of the C_{20} methyl derivative XXXIX which oxidized readily to allopregnane-3(β),17(β)-diol-20-one (substance L) (XL) with periodic acid (90). This hydroxy ketone was also prepared by meth-



XXXIX



XL

ods analogous to those employed in the partial synthesis of 17(β)-hydroxyprogesterone (79, 80).

The series of four possible allopregnane-3(β),17,20-triols has been completed with the preparation of the 17(α),20(β)-isomer (91). Oxidation of androstane-3(β),11-diol-17-one to the 3,17-diketone in 64 per cent yield by Oppenauer's method using aluminum phenolate demonstrates a convenient means of converting 3,11-dihydroxy steroids to 3-keto-11-hydroxy derivatives (92). Another example of this preferential oxidation is the analogous conversion of urane-3(β),11-diol to urane-11-ol-3-one using aluminum isopropylate (93).

URINARY STEROIDS

Examination of the urine of a girl suffering from adrenal virilism yielded a new androstene-3(α)-ol-17-one in which the location of the

double bond is limited to the 6,7-, 7,8-, 9,11-, or 11,12-position. The occurrence of significant quantities of androsterone, etiocholanone-3(α)-17-one, dehydroisoandrosterone, and 3,5-androstadiene-17-one in concentrates of such urine was confirmed. The excretion of dehydroisoandrosterone by this patient was very high. The new androstenolone and the androstadienone are believed to arise by dehydration of precursor compounds during the acid hydrolysis of the urine (94). Isoandrosterone, first isolated from the urine of women with adrenal cortical pathology (95, 96) has now been shown to be a constituent of the urine of normal women and of both male and female patients with cancer (97). The pregnane-3(α),17,20-triol which occurs in the urine of women with the adrenogenital syndrome (95) has a different configuration than the pregnane-3(α),17,20-triol obtained from the osmic acid hydroxylation of 17-pregnene-3(α)-ol (98).

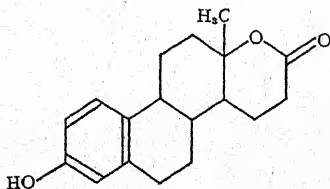
In the course of work pointing to the development of a systematic method for the study of the urinary excretion of steroid compounds the following steroids were again isolated from normal male urine: androsterone, etiocholanone-3(α)-ol-17-one, isodehydroandrosterone, cholesterol, and pregnane-3(α),20(α)-diol (99, 100). The last-named compound has also been isolated from the urine of pregnant chimpanzees (101), which is of particular interest since it was not detected in the urine of pregnant Rhesus monkeys (102).

A new hydroxy ketone was isolated from the urine of pregnant mares. Its structure was shown to be that of a 5,7,9-estratriene-3-ol-17-one. It is considered to be an intermediate in the metabolic reduction of equilenin to the estranediols (103). The estranediols, however, have been obtained thus far only from the urine of pregnant women (104). The triol first isolated from mare's pregnancy urine by Haslewood, Marrian & Smith (105) was suggested by Odell & Marrian (106) to be a pregnane-3(α),6,20-triol. Later Marker & Wittle (107) obtained evidence that the triol was an allopregnane-3(α),-16,20-triol. This formulation has been further supported by the conversion of the triol to the known 16-allopregnene-3,20-dione by oxidation with aluminum isopropylate in cyclohexanone, dehydration at the 16,17-position occurring under the conditions of the reaction. An effort to obtain the urinary triol by the degradation of *epitigogenin* was unsuccessful, the allo-pregnanetriols obtained differing from the urinary triol presumably by the configuration around C_{16} and C_{20} . The results are interpreted as indicating that the configuration around C_{16} in the urinary triol is opposite to that in *epitigogenin* (108).

METABOLIC TRANSFORMATIONS

Estrone to the extent of 2.5 per cent of the administered α -estradiol was recovered from the urine of both normal and spayed female guinea pigs (109). That the ovary and uterus do not have a more specific effect on the percentage conversion is not surprising when one considers that the concentrations of estrone and α -estradiol are much higher in horse testes than in any other tissue (110). In fact 6.5 per cent of administered α -estradiol was recovered as crystalline estrone from the urine of a normal man. About 4 per cent was recovered unchanged and the remainder, about 90 per cent, was not accounted for (100). Estrone (2.6 per cent) and β -estradiol (12.1 per cent) were isolated from the urine of normal and ovariectomized-hysterectomized rabbits receiving α -estradiol (111, 112). Only estrone was isolated from the urine of the same type of experimental subject when estrone was administered (112). The simultaneous administration of progesterone did not influence the percentage conversion of α -estradiol to estrone or β -estradiol in the rabbit. Pregnanediol-3(α),20(α) was isolated from the urine in an amount equivalent to 10 per cent of the administered progesterone (comparable to the percentage conversion in man) but in the rabbit pregnanediol is not eliminated in conjugation with glucuronic acid (111). Likewise in the bull pregnanediol is not excreted in conjugation with glucuronic acid (113).

In an effort to gain a clue to the metabolic degradation of estrone its oxidation with hydrogen peroxide in alkaline solution was studied (114). One product of such oxidation was a crystalline lactone which is fourteen times less active estrogenically than estrone and which was assigned the structure XLI. Estrone is readily inactivated by



XLI

laccase but no degradation products have been identified. Contrary to earlier reports estrone is not inactivated by tyrosinase (115).

An estrone-protein complex was prepared by converting estrone

to its *p*-aminophenyl ether and coupling the diazotized amine to casein. The ether was estrogenic at 10 μ g, the azoprotein at 830 μ g (116).

Isoandrosterone (117) has been added to androsterone and etiocholane-3(α)-ol-17-one as excretion products of administered testosterone in the human male. The demonstration of the formation of both etiocholane-3(β)-ol-17-one and its C_8 epimer, isoandrosterone, by the catalytic reduction of 5-androstene-3(β)-ol-17-one (118) is of interest in connection with the possibility of isodehydroandrosterone being an intermediate in the reduction of testosterone *in vivo*.

The view that the urinary isodehydroandrosterone is a reduction product of a Δ^4 -3-ketosteroid (119) has been criticized primarily because of the lack of any analogous biological reduction of a Δ^4 -3-ketosteroid to a Δ^5 -3-hydroxysteroid, the earlier demonstration of the bioconversion of cholestenone to cholesterol (120) being held unconvincing (94, 121). In support of the former view it has now been found that a dog fed and injected with 4-dehydrotigogenone (the analogue of cholestenone in the sapogenin series) excretes in the feces at least 3.3 per cent of the compound as diosgenin, the analogous Δ^5 -3-hydroxy sapogenin (122, 123).

Although fermenting yeast will not reduce the α,β -unsaturated ketone grouping in testosterone or 4-androstene-3,17-dione (124) it will readily do so in 1-androstene-3,17-dione or 1-androstene-17-ol-3-one, isoandrostanediol being the only product isolated in either case (125).

CHEMICAL ASSAY

The importance as well as the difficulties of the chemical determination of the various urinary steroids is attested by the numerous papers which have appeared in this field. Several improvements have been made in the apparatus for initial hydrolysis and extraction of the urinary steroids (126, 127), the most significant one allowing simultaneous hydrolysis and extraction (128). The adsorption procedures of Callow (129) have been adapted to the routine determination of the twenty-four-hour excretion of alcoholic and nonalcoholic 17-ketosteroids by applying the Zimmermann color reaction before and after filtration through a column of alumina. Only the alcoholic 17-ketosteroids are adsorbed (130, 131). Separation of the 17-ketosteroids with Girard's reagent T and the alcoholic 17-ketosteroids as the half succinic acid esters was recommended as a means of eliminating interfering chromogens (132). The conditions necessary for

obtaining optimum results with the Zimmermann color reaction (*m*-dinitrobenzene and potassium hydroxide) have been the subject of considerable study (133, 134, 135, 136), some workers employing a special tube in which to prepare the color reaction mixture (137).

Detailed methods suitable for clinical use were described for the photometric determination of estrogens, estriol being determined separately. A modified Kober color reaction was used (138). The addition of powdered zinc during the acid hydrolysis of the urine was found to increase the yield of estrogens (139). Solubilities of estrone, estriol, α -estradiol, and equilenin were recorded for a number of solvents (140) and dibutyl ether was recommended as the solvent of choice for the extraction of urinary androgens in colorimetric assay work (141).

Kleiner (142) has described a sensitive color reaction for the natural estrogens based on their ability to couple with phthalic anhydride. Estrone, α -estradiol and estriol give a deep pink color with a greenish yellow fluorescence when coupled in the presence of stannic chloride. The reaction is sensitive to 0.25 μ g. of estrone. The optical properties of the colored products differ from those of products formed from other phenolic substances. It was found that the sensitivity of the Kober color reaction for estrogens could be increased by the substitution of guaiacolsulfonic acid for phenolsulfonic acid (143). The addition of copper or iron salts to the reaction mixture makes the test specific for testosterone and 4-androstene-3,17-dione, these two compounds giving a bright green color of approximately equal intensity. Other known androgenic steroids do not interfere (144).

In the Venning method (145) pregnanediol is determined gravimetrically as sodium pregnanediol glucuronide. It has now been found that the pregnanediol content of urine can be determined by acid hydrolysis and simultaneous extraction with toluene followed by the direct crystallization of pregnanediol after separating some impurities. This simple procedure is also recommended for preparative purposes (146). A titrimetric method has also been devised which gives values comparable to those obtained by the Venning procedure (147). The pregnanediol conjugate is isolated through its very insoluble lead salt and the pregnanediol estimated by the reducing capacity of the glucuronic acid freed by acid hydrolysis.

A gravimetric method for the determination of ketosteroids was described by Hughes (148). It depends on the conversion of the ketone to its water-soluble hydrazone using Girard's reagent T and

precipitating the hydrazone with mercuric iodide. The mercuric iodide-hydrazone complex, the exact composition of which has not been determined, is filtered off, dried and weighed. Quantitative results were obtained in determining the carbonyl groups in 20 mg. quantities of estrone and progesterone.

The Heyrovsky polarographic method of analysis has been applied to the determination of ketonic steroids both in pure solution and in urinary extracts (149, 150, 151, 152). The results on urinary extracts closely parallel those obtained with the Zimmermann colorimetric method. The basis of the application is as follows. The 3- and 17-carbonyl groups of the steroid nucleus are not reduced within the measurable range of potential. Δ^4 -3-Ketosteroids reduce within the measurable range but none are known to be present in urine. The 17-ketosteroids are converted to the 17-hydrazone with Girard's reagent T which do reduce in the measurable range. The corresponding hydrazones of saturated 3- or 20-steroids do not interfere. Therefore, by first separating estrogenic 17-ketones from the urine extract, the total 17-keto androgens can be determined. Oppenauer oxidation of a portion of the urinary extract converts isodehydroandrosterone to 4-androstene-3,17-dione. Its concentration is measured by means of the reduction potential of its 3-hydrazone which is characteristic for the hydrazone derivatives of Δ^4 -3-ketosteroids.

The infrared absorption spectra of a number of steroid hormones and related compounds have been measured. They are reported to be unique for the individual compounds with the exception of space isomers. The technique is applicable to the analysis of biological fluids (153).

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THE WATER-SOLUBLE VITAMINS

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INTRODUCTORY REMARKS

In the limited cross section of research covering a single year it is difficult to assess the direction of the still increasing flow of investigative work pertaining to the water-soluble vitamins. Some new or heretofore less prominent trends have, however, become easily discernible during 1941. In reviewing this development it seems appropriate first to discuss one or two general aspects of the problems involved.

Definition of a vitamin.—The gradually extended scope of vitamin research, especially that dealing with the vitamin B complex, touches more and more on closely related but hitherto separate fields. Thus the question of what should be considered a vitamin needs to be viewed from a wider angle. A few years ago the following definition was proposed (1):

“Vitamins are food constituents of organic origin, minute quantities of which have specific biologic effects and lack of which in the food produces pathologic disturbances, occasionally only under special conditions.”

In the light of recent research two objections can easily be raised to this definition:

(a) Should choline, because it is used not in “minute quantities” but in relatively high doses, be excluded from the group of water-soluble vitamins and should it, in consequence, be called only a “dietary essential” (2)? If the answer is in the affirmative, why should ascorbic acid, pantothenic acid, and nicotinic acid, all of which are also administered in rather large doses, be called vitamins, and what is the objective reason for the erection of such an artificial barrier? And if choline should be considered a vitamin—a view with which this reviewer concurs—why, then, should methionine, which as a rule has choline-like properties, be excluded from the group of vitamins? In further consequence there would then be raised the problem of classifying essential amino acids as close relatives of vitamins.

(b) In the light of recent studies it is unnecessary to make the classification of a vitamin depend on its character as a food constituent. Some vitamins, such as various members of the vitamin B complex, can be synthesized in the rumen of certain animals by bacteria (3 to 8). Moreover, it appears very probable that biotin, at least in Mammalia, is supplied mainly by the activity of the intestinal bacteria (9). The same process might also be assumed in the case of "folic acid" (10), the most recent member of the vitamin B complex. The presence of these factors in the ingested food would not necessarily be a prerequisite for adequate nutrition.

In recent investigations which touch on the same problem, sulfa-guanidine (11, 12) was fed to rats in order to detect the lack of synthesis of unidentified essential nutrilites by the intestinal bacteria. These experiments will be discussed later in connection with *p*-amino-benzoic acid.

Microorganisms in vitamin research.—During the past few years much attention has been paid to microorganisms, not only as possible synthesizers of vitamins but also as easily available test objects whose vitamin requirements can be observed with important saving in time, cost, and labor. Through experiments of this kind it has become evident that, generally speaking, the same growth substances, foremost among which are members of the vitamin B complex, needed by animals, are concerned also in the growth of all living cells and, in particular, of bacteria and yeast cells (13, 14). R. J. Williams and his collaborators (15) have utilized these bioassays for an extensive study of the distribution of vitamins in different tissues and for other special problems. Undoubtedly these methods have a wide field of practical applicability, and in the future they will certainly play an increasingly important role in vitamin research. It should be borne in mind, however, that they also have their limitations. Furthermore, the data recently made available are from experiments not carried out on a sufficiently large scale and need extensive amplification.

The specificity of the growth response is generally less pronounced in microbiological assays. Compounds that are chemically closely related to some vitamins, such as nicotinuric acid (16) or the thiazole and pyrimidine portions of the thiamin molecule (17, 18, 19), do produce growth in bacteria and increased fermentation in yeast cells similar to the effects of nicotinic acid and thiamin, respectively, but they are ineffectual in treatment of canine blacktongue (20) or in thiamin-deficient animals (17). On the other hand, cocarboxylase,

which is generally regarded as the most effective end-form of thiamin in cell metabolism, has practically no influence on yeast cells in the growth test (19).

Substances present simultaneously with vitamins in blood, tissue, and food extracts may enhance the action of vitamins in microbiological assays and thus, to some extent, falsify the quantitative results obtained. Starch (21), and some unknown blood constituent (22), may vitiate the quantitative tests for riboflavin; and a heat-labile factor present in blood (23) may invalidate the test for thiamin.

The microbiological tests share with the chemical methods the difficulty involved in preparing extracts from foodstuffs without loss of vitamins, in such a way that the extracts may, at the same time, contain the vitamins in biologically active form, equivalent in quantity and quality to the corresponding vitamin-like substance utilized in the animal body after ingestion. Williams and his collaborators (15) relied mainly on the autolytic liberation of the members of the vitamin B complex, but they themselves point out (24) that the values for pantothenic acid thus obtained were in many instances lower than those based on animal tests, a result that might be explained by incomplete extraction in the bioassays. These workers have recently extended their previous investigations on biotin and have reached the conclusion (25) that acid hydrolysis is the best method for liberation of biotin from animal tissues, which confirms the work of others (26, 27). Autoclaving at high pressure, with or without the addition of acid, can be applied with equally satisfactory results (26).

By painstaking studies, these few inadequacies of the microbiological methods have already been overcome to a large extent and the shortcomings still present are by no means insurmountable.

Concurrence of vitamin-deficiency diseases.—It is a truism, repeatedly stated in the past as well as during the year 1941 in experimental and clinical investigations, that, in view of the simultaneous presence of the members of the vitamin B complex in foodstuffs, the occurrence of a single deficiency involving only one of the B vitamins is distinctly unusual. Even in the case of a multiple deficiency, however,

rarely are fully developed symptoms of more than one vitamin deficiency disease encountered. Rather one disease gets the upper hand and seems to relegate to a latent position other diseases the etiologic conditions of which are provided in the lack of the corresponding vitamin in the diets (1).

This phenomenon, called "concurrence of vitamin-deficiency diseases"

(1), indicates that it is perfectly possible that cure of the prevailing deficiency disease by the specific vitamin involved may often activate a second deficiency disease, the etiologic manifestations of which are suppressed by the predominant deficiency.

Relevant observations have been reported during the past year in experimental (28) and clinical (29, 30) studies. They also have a distinct bearing on the problem of disturbed equilibrium in the dietary supply of vitamins and, in further consequence, on the problem of fortification of foodstuffs with vitamins (28, 31, 32).

In the concurrence of vitamin-deficiency diseases the interaction between the "primary" disease and the suppressed, latent disease is apparently mutual, the latter having also a mitigating effect on the former. This assumption is borne out by the experimental studies of Alexander (33) on the production of Wernicke's encephalopathy in pigeons, which he found as a complication of beriberi only after large supplies of all B vitamins except thiamin had been given but which he rarely observed in pigeons fed an entirely vitamin-free diet.

THIAMIN

Inactivation of thiamin by a substance present in raw fish.—One of the outstanding results of thiamin research in 1941 was the speedy unraveling of the puzzling endemic occurrence of Chastek paralysis on fox ranches (34, 35). The mortality was reported to be especially high among nursing pups. Clinically the syndrome is characterized by hyperesthesia, anorexia, weakness, ataxia, spastic paralysis, and rapid death within 48 to 72 hours after onset of the neurological symptoms. On post-mortem examination vascular lesions were found bilaterally in the brain, located principally in the paraventricular gray matter. These lesions were identical in appearance and localization with those of Wernicke's hemorrhagic encephalopathy in man and in pigeons (33). The disease was seen only in foxes kept on farms where uncooked fish was included in the ration in a level of 10 per cent or more. It could readily be produced experimentally in foxes fed a diet that contained fresh carp. Addition of large amounts of thiamin to the diet prevented the outbreak of the disease, and it was therefore suggested (34) that some constituent of raw fish has the capacity to inactivate thiamin. The disease can also be produced in chicks or caused to occur again in foxes which have recovered from a previous attack (36). The inactivation of thiamin by raw fish takes place within the feed mixture and may be either an enzymatic degra-

dation (36, 37) or may be due to the formation of a "thiamine-anti-thiamine complex" (37). Woolley (37) reported that the active ingredient in fresh carp was heat labile, not dialyzable and could be extracted with 10 per cent solution of sodium chloride. These extracts were found (37) to be active in suppressing the growth-promoting effect of thiamin on yeast cells (*Endomyces vernalis*).

Assay and distribution.—Different modifications of the chemical, thiochrome method have been proposed which, it has been claimed (38, 39, 40), give satisfactory results. In assays of thiamin in urine, the importance of a preliminary extraction with isobutyl alcohol (41) has been emphasized (42). Ingestion of large amounts of nicotinic acid results, in man, in an apparent increase in excretion of thiamin in the urine, as determined by the thiochrome method (43). Thus, a high intake of nicotinic acid and simultaneously low urinary output of thiamin could give rise to a serious error in the use of the thiochrome method.

Microbiological methods, such as the yeast-fermentation method introduced by Schultz, Atkin & Frey (44) and the yeast-growth test recently proposed by R. J. Williams and his collaborators (19), have been found reliable in assaying food products, tissue extract, blood, and urine for thiamin. The values obtained when urine is assayed by the fermentation method measure the sum of the thiamin and the pyrimidine breakdown products present. After oxidation of the free thiamin (as in the thiochrome test), repetition of the fermentation assay will give values for the pyrimidines alone and thus, by subtraction of the latter from the total, the values for free thiamin (18).

A modification of the rat-growth test has been recommended (45). In the improved method, traces of thiamin usually present in the experimental ration are destroyed by treating the casein and the liver extract with sulfite, when the liver extract is used as the source of the other members of the B complex.

By means of the various chemical and biological assays the thiamin content has been determined in various foodstuffs, such as rice (46), cereal products (39, 47, 48), bread (49), and peanuts (50). In cow milk only about 60 per cent of the total thiamin present occurs in a free state (51). From the nondialyzable remainder, which is probably a thiamin-protein compound, thiamin can be freed by the combined interaction of taka-diastase (or a potent phosphatase) and a proteolytic enzyme, for instance, papain (51).

Assessment of thiamin status.—In normal subjects the thiamin

values in blood determined by the fermentation method have been found to be between 3.1 and 9.2 $\mu\text{g.}$ per 100 cc., with an average of 5.4 $\mu\text{g.}$; values below 3 $\mu\text{g.}$ by this method appear to indicate a state of thiamin deficiency, such as may exist in cases of peripheral neuropathy or in Wernicke's syndrome (23). In the blood of infants and young children there is, normally, a level of about 10 $\mu\text{g.}$ of cocarboxylase per 100 cc. The values become lower in the case of prolonged or acute illness (52, 53).

Urinary excretion of thiamin is diminished in a person deficient in thiamin (54, 55). In a large number of investigations, the saturation test, with various but not essential modifications, has been found to be the most reliable way to assess a possible deficiency of thiamin (56 to 61). In a patient suffering from chronic alcoholism with neuropathy (62), in persons with cardiac failure (63) and in pregnant and lactating women (64), the test was positive, showing the need for more thiamin, but it was negative in persons with hepatic disease without accompanying neuropathy (65). In the positive test in cardiac failure the nonspecific effect of impaired circulation may play an important part.

In eight older subjects (four men and four women in the seventh or eighth decades of life) the response in urinary excretion to increased intake of thiamin was similar to that of normal younger people (66). Even great alteration in the ratio of fat to carbohydrate in diets having the same (adequate or low) thiamin content did not significantly affect the urinary output of thiamin (67).

Recently, in the diagnosis of thiamin deficiency there has also been utilized the rise in content of bisulfite binding substances and in particular of pyruvic acid in blood and urine in animals (68, 69, 70), or in children and adults (53, 71, 72, 73) inadequately supplied with thiamin. The average content of pyruvic acid, which is more reliable (53) as a measure of thiamin deficiency than is the total amount of bisulfite binding substances, is about 1 mg. per 100 cc. of blood in both children (72) and adults (73). In patients with cardiac failure the blood pyruvic acid level has been found to be elevated (74).

Of practical diagnostic value is the study of the changes in content of pyruvic acid in the blood following ingestion of glucose. In normal subjects the blood pyruvic acid curve shows a short, steep elevation that reaches its maximum at the end of one hour and returns to the normal fasting range within three hours. In conditions associated with thiamin deficiency the blood pyruvate curve after ingestion of

glucose starts at a higher range and is abnormally elevated for a prolonged period of time (73, 75).

Effect of processing on thiamin.—When husked unpolished rice is steamed under reduced pressure for from two to four hours at temperatures between 40° and 60° and then dried, it retains a large proportion of its thiamin content even after it has undergone subsequent polishing operations, because the thiamin of the outer coat has penetrated the grains during steaming (76). Evaporated milk retains only from 40 to 61 per cent of the thiamin contained in raw milk before evaporation (77). During the baking process, white bread, as well as bread enriched with thiamin or yeast, loses not more than an average of 10 per cent of the thiamin originally present (78, 79). This loss takes place almost exclusively in the crust (79).

Physiology and pharmacology.—In plants, thiamin appears to function as an activator for indolebutyric acid (80).

Addition of thiamin to the culture medium for *Drosophila melanogaster* causes the larvae to develop at a more rapid rate. The whole larval period is shortened by twenty-four hours by such addition (81).

With regard to the often-suggested relationship of thiamin to the functions of the gastrointestinal tract, it is of interest to note that no serious damage to the secretory cells of the gastric mucosa has been found in rats suffering from extreme deficiency of thiamin (82). Vitamin B₁ is stable in normal gastric juice from pH 1.5 to 8, as well as in gastric juice from patients with achlorhydria, but it is destroyed to a large extent (50 to 90 per cent) *in vitro* after incubation for sixteen hours with bile (83). In patients with achlorhydria, the absorption of thiamin seems to be unimpaired (84).

Thiamin is not a dietary essential for ruminants but can apparently be synthesized in the rumen of sheep and cows even when they are fed diets low in thiamin (3, 6). The data obtained indicate, however, that synthesis of thiamin in the rumen, apparent four hours after feeding, may decrease twelve or sixteen hours after feeding, perhaps owing to bacteriological destruction (3).

Whereas perfusion of thiamin has no effect on the isolated intestine of a normal rat, it is claimed (85) that the peristalsis of isolated intestinal loops obtained from a thiamin-deficient rat is increased and that the rate of progressive tonus loss is retarded under the influence of thiamin *in vitro*. This observation has been connected with better utilization of acetylcholine by the isolated intestine of thiamin-deficient animals, when thiamin is present (85). In this respect, however, the

reports published recently are rather contradictory. Normal amounts of acetylcholine have been found in brain and heart of beriberic pigeons (86) and the cholinesterase values were described as several times higher in thiamin-deficient than in normal pigeons (87), but much lower, at least in the liver (1:6), in thiamin-deficient rats than in normal control animals (88). Thiamin deficiency makes rats more resistant to nicotine (89). The adrenal cortex undergoes both relative and absolute hypertrophy (90) in dogs suffering from acute deficiency of thiamin, and the concentration of steroid compounds is increased (91).

In the bull the adrenal cortex is more than seven times as rich in thiamin as the medulla, while in the cow the medulla is 1.9 times as rich as the cortex (92). These interesting sexual differences found in preliminary experiments need further amplification.

In experimental hyperthyroidism of dogs, produced by feeding thyroid, loss of appetite becomes quickly manifest when the B vitamins, for example in the form of yeast, are removed from the diet. The appetite can be restored within twenty-four to forty-eight hours by one injection of 2 mg. of thiamin. The weight of the hyperthyroid dogs could also be maintained as long as no deficiency of the B vitamins occurred (93).

On the basis mainly of subjective observations of cyclists and football players (94) and of work-test experiments (95) it has been claimed that physical performance greatly increases if thiamin (with or without ascorbic acid) is given during exertion. In contrast to these claims, no objectively favorable effect on the response to intensive exercise could be substantiated when daily supplements of 5 mg. of thiamin and 100 mg. of ascorbic acid were added to the standard United States Army ration. Observations were controlled by comparative determination of heart rate, diastolic and systolic sizes of the heart, stroke output, blood pressure, and blood hemoglobin, lactate, and sugar (96).

Thiamin enabled dogs anesthetized with ether, in which shock had been induced by hemorrhage, to live significantly longer than untreated animals; at the same time thiamin produced a distinct sustained rise in blood pressure (97) and a definite fall in elevated blood keto-acid values (98).

Thiamin and abnormal behavior.—As a special approach to psychological studies, the behavior pattern of the albino rat characterized by epileptoid seizures when subjected to auditory stimulation was studied (99) under different conditions. In preliminary investigations

results were reported (100, 101) which were interpreted as due to the protective effect of members of the vitamin B complex, in the first place by thiamin. The opinion was expressed that results of experiments of this kind will be "applicable to the detection and quantitative evaluation of certain nutritional deficiencies of marginal type" (100).

Another example of the relationship of thiamin to problems of experimental psychology is found in the difference in the speed of acquisition of a conditioned eyelid response in thiamin-deficient albino rats as compared with normal litter mates. Response in the deficient animals was significantly poorer than in the controls (102).

Self-selection of diet and thiamin.—In experiments on rats maintained on a self-selection diet of all necessary food constituents, ingestion of thiamin particularly stimulated the appetite for carbohydrates (103). In similar investigations conducted with adult persons, no correlation was demonstrable between intake of thiamin and carbohydrate, on the one hand, or between thiamin and the total caloric intake, on the other (104). The intake of thiamin in self-selection experiments showed great variation among individual members of the experimental group but exceeded the theoretical requirement in most instances (104).

Experimental thiamin deficiency in animal and man.—When young pigeons are fed a completely thiamin-free diet by tube in quantities sufficient to prevent large loss in weight, an acute deficiency ensues that is characterized by severe opisthotonus. The main symptoms in chronic hypovitaminosis are ataxia, leg weakness, and cardiac failure (105). Signs of cardiac failure which react favorably to thiamin are also encountered in dogs made chronically deficient in thiamin. Microscopic examination of the myocardium reveals many small areas of necrosis (106). Prolonged low intake of thiamin (2 μ g. per kilogram of body weight daily) with an otherwise adequate diet leads in dogs to neurological disturbances that are characterized clinically by spasticity of the hind legs, staggering gait, unsteadiness, vomiting, and, on microscopic examination, by degenerative changes of the peripheral nerves and of the posterior columns of the spinal cord (107). Thiamin is required also by the growing pig, approximately in an amount of 1 mg. per day per 100 lbs. of body weight (108, 109).

Objective and subjective symptoms of a deficiency state developed early in human volunteers maintained for from 132 to 196 days on a diet containing only 0.4 to 0.45 mg. of thiamin daily (110). The subjects became morose, depressed, fearful, irritable, uncooperative,

and lacked any interest in working. It was claimed that they reacted immediately to higher intake of thiamin (110). The evaluation of mainly subjective symptoms is open to criticism. The minimum on which man can exist is 0.6 mg. of thiamin daily. To function efficiently he needs twice this amount, and for safety around 2 mg. daily. This upper limit is seldom reached in the usual American diet (111).

Clinical use of thiamin.—While in previous publications thiamin was often indiscriminately recommended for peripheral neuritis of varying origin, recently a more critical note has been sounded. It has been stated (112) that peripheral neuropathy which involves a single nerve, which is not bilateral or symmetrical or which does not affect first and foremost the lower extremities, is not, as a rule, due to thiamin deficiency or to it alone. Peripheral neuropathy which fulfills all these conditions and responds to thiamin is often found in alcoholics (113) but is by no means frequently found in persons with diabetes mellitus (114, 115) or in diphtheritic patients (116, 117). Even in the case of the peripheral neuropathy of alcoholics (118) and of pregnant women (119) or in trypanamide amblyopia (120), thiamin therapy may fail to produce beneficial results. Thiamin has likewise no significant effect on the general course of diabetes mellitus (121, 122) or on the morphine abstinence syndrome in man (123).

More satisfactory results were reported on the use of thiamin in the treatment of migraine (124) and the nausea and vomiting occurring in radiation sickness (125). Slight improvement was also recorded (126) of residual neural disturbances in patients with pernicious anemia who were treated with liver after intramuscular administration of thiamin.

Concerning other neuropsychiatric disorders, administration of thiamin was found to be of great value and could be considered life-saving in the treatment of Wernicke's syndrome (113, 127, 128, 129, 130), in analogy to the corresponding experiments made on pigeons and foxes (33, 34, 35). In contrast, delirium tremens (131) and Korsakoff's syndrome in alcoholics (113), although often combined with peripheral neuropathy of the B₁-deficiency type, appear to present no adequate indication for administration of thiamin, even in combination with nicotinic acid.

Untoward effects of thiamin.—There have been reported (32, 132, 133, 134) very few cases of sensitization to thiamin with severe constitutional reaction and collapse after its injection. The sensitization was due either to thiamin itself, in which case the reaction could be

elicited by injection of an aqueous solution of chemically pure thiamin (132), or to the preservative added to the thiamin solution (112). The intradermal test for detection of possible sensitivity should be performed with a very dilute solution of thiamin (5 mg. per cubic centimeter), as higher concentrations may provoke nonspecific wheal formation (135).

NICOTINIC ACID AND NICOTINAMIDE

Methods of determination.—The chemical methods widely used to determine the content of nicotinic acid depend primarily on the rupture of the pyridine nucleus and subsequent coupling with an aromatic amine (aniline, *p*-aminoacetophenone) to form a colored compound (136, 137).¹ During 1941 various modifications have been proposed with special reference to alkaline or acid hydrolysis, to decolorization of the extracts and to the question of interference of colored contaminants and their elimination (136, 137, 138, 139). A very satisfactory chemical method for the quantitative estimation of nicotinic acid in animal tissues has been devised by Dann & Handler (140, 141).

In the selection of the test organism to be used in the quantitative microbiological determination of nicotinic acid, nicotinamide, and related substances, among the latter being coenzyme I (diphosphopyridine nucleotide) and coenzyme II (triphosphopyridine nucleotide), it should be borne in mind that all these compounds do not always have equivalent growth-promoting effects on bacteria. In tests on a series of microorganisms (142) the ratio of activity of amide to acid varied from one tenth (*Bacillus diphtheriae*) to infinity (organisms of the *Pasteurella* group). *Hemophilus influenzae* must be supplied with the whole coenzyme molecule (142). Satisfactory quantitative results were obtained by microbiological assay with *Shigella paradysenteriae* (143) and *Lactobacillus arabinosus* (16, 144, 145). The method using *Shigella* has been employed mainly for determinations in blood, urine, serum, or spinal fluid and the assay with *Lactobacillus* for tissue extracts won by autolysis. Comparative values obtained by microbiological and chemical methods are in good agreement (145). It should, however, be pointed out that the assays compared were not carried out on the same sample.

¹ It should be borne in mind that, with few exceptions, only papers which appeared during 1941 are included in this review. Methods referred to, however, not only under Nicotinic Acid but also under other members of the vitamin B complex, originated in many instances earlier than 1941.

Sulfapyridine and metabolism of nicotinic acid.—It has been claimed that the pyridine group of the drug sulfapyridine specifically inhibits the activity of the enzymes associated with nicotinamide and related compounds and therefore adds substantially to the bacteriostatic activity of the sulfanilamide moiety. Inasmuch as the thiazole group of sulfathiazole is isosteric with the pyridine group in sulfapyridine, the same mechanism should apply also to sulfathiazole (146). These observations have not been confined to bacteria but have been demonstrated also in animals. Blacktongue in dogs failed to react to nicotinic acid in the presence of sulfapyridine (147). Unexplained is the further finding that liver may counteract the inhibitory effect of sulfapyridine and cure blacktongue (147).

These experiments are, in the last analysis, connected with the therapeutic effect of nicotinic acid (nicotinamide), which would seem to center around the synthesis of coenzymes in the cells. In the light of special investigations the opinion was expressed (148) that some specific protection against cozymase breakdown may also play a part in the therapeutic effect of nicotinamide.

Distribution of nicotinic acid in foodstuffs.—By chemical and microbiological methods the distribution of nicotinic acid in different foodstuffs has been determined (137, 139, 140, 145, 149, 150, 151). Liver, and to a less extent kidney and yeast, were found to be the richest sources of nicotinic acid. It is of special interest to note that milk contains only small amounts of nicotinic acid, an average of 80 μ g. per 100 cc. (139), whereas liver and hog muscle contain about 15 mg. and 5 mg. per 100 gm. of fresh tissue, respectively (149, 151). Cooking and commercial processing of meat products cause only minor losses of nicotinic acid (151).

Nicotinic acid and related compounds in blood, urine, and animal tissues.—No significant differences in the content of nicotinic acid (determined chemically) could be detected in the blood of normal people and of those suffering from pellagra. "Tolerance curves" of nicotinic acid or nicotinamide were likewise without conspicuous value in elucidating conditions of the deficiency (152). In analogy to these observations, the coenzyme I content of erythrocytes is on practically the same level in normal subjects as in pellagrins at varying stages of the disease. Administration of nicotinic acid to pellagrins, as well as addition of nicotinic acid or nicotinamide to defibrinated blood *in vitro*, led to marked increase in the coenzyme I content of erythrocytes (153). Administration of quinolinic acid, of pyrazinemonocarboxylic

acid, or of pyrazine-2,3-dicarboxylic acid had no effect under similar conditions and none of the three acids was effective in curative trials on blacktongue (154).

In contrast to the behavior of the coenzyme content of blood, there is a significant decrease from normal in the values in the liver and muscle of dogs suffering from blacktongue (155) and in the muscle of pellagrins (153). The content of unbound nicotinic acid in the liver remains uninfluenced by blacktongue (155). Addition of nicotinic acid to the diet of pellagrins produces a rise in the content of coenzyme in muscle just as it does in erythrocytes (153).

All the nicotinic acid of the kidney cortex and muscle exists as part of the nucleotide molecules, while in liver 58 per cent of the nicotinic acid exists in some other form (156). In experimental hyperthyroidism of rats fed a diet low in nicotinic acid the coenzyme content of liver and kidney cortex was distinctly reduced but could be restored to normal by increasing the intake of nicotinic acid (157).

The urinary excretion of nicotinic acid in pellagrins is not significantly different from that in normal subjects (152, 158). Derivatives of nicotinic acid and trigonelline excreted with the urine account for from 30 to 50 per cent of the dietary nicotinic acid in man and dog (159). The opinion has been expressed (152) that the urinary excretion of trigonelline may be a good measure of nicotinic acid nutrition. On the basis of experiments with dogs, the saturation test has also been recommended for assessment of the nicotinic acid status (159).

Synthesis of nicotinic acid by the rat and by the chick.—By comparing the amount of nicotinic acid in the body of the growing rat with the intake of nicotinic acid, it was concluded (160) that nicotinic acid is synthesized within the body of the rat. Moreover, from the fact that administration of nicotinic acid did not improve the diminished rate of growth of rats brought about by ingestion of sulfaguanidine, the point of attack of which is supposedly the intestinal flora (11, 12), it was further inferred (160) that the synthesis of nicotinic acid is "not due to the symbiotic activity of microorganisms in the intestine." Of course it is still possible that sulfaguanidine, through its effect on the intestinal flora, deprives the body of several other essential nutritives in addition to nicotinic acid, which then cannot be replaced by nicotinic acid alone. These doubts, however, certainly do not apply to conditions in the hatching chick, which contains ten to twenty times as much nicotinic acid as the unincubated egg (161, 162). Here the syn-

thesis must have taken place within the tissues and organs of the chick embryo.

Pellagra and fluorescent substances in urine.—Fluorescence in the urine of pellagrins was in the past erroneously attributed to specifically increased excretion of porphyrin. The presence of porphyrin in the urine is not an essential feature of pellagra and does not in any way aid in the diagnosis of the disease (163).

A bluish fluorescent substance (F_1), the composition of which has not yet been established, is regularly found in high concentration in the urine of pellagrins and is normally present only in relatively small amounts. The reverse is true of a second equally bluish fluorescent substance (F_2), also chemically unidentified, that is present in the urine of normal persons and is materially reduced in amount in pellagrins. Administration of nicotinic acid or other pyridine compounds with antipellagra activity causes a reduction in the amount of F_1 and reappearance of F_2 in the urine of pellagrins (164, 165, 166).

Clinical use of nicotinic acid and nicotinamide.—The beneficial effect of nicotinic acid and nicotinamide in pellagra has remained uncontested. In the so-called "encephalopathic syndrome" which may be observed in persons suffering from acute deficiency of nicotinic acid, often without any other concomitant sign of pellagra, administration of nicotinic acid (amide) has been found to be equally highly effective (113, 167). In psychosis of longer duration, the results were not so satisfactory (113), probably because the changes became irreversible.

In Ménière's syndrome (168) and in the mild psychotic conditions of old age, such as senile encephalomyelosis (169), nicotinic acid, often in combination with thiamin or liver extract, gave good therapeutic results.

Vasodilatation, evidenced by the flushing of the skin after medication with nicotinic acid, may extend to the cerebral vessels, and thus by improved circulation add to the therapeutic power of nicotinic acid in diseases of the brain. This possibility has been both affirmed (170) and denied (171).

RIBOFLAVIN

Methods of determination.—During 1941 various improvements in the older chemical, physical, biological, and bacteriological methods used to assay riboflavin have been reported. A new method, the polarographic determination of riboflavin in natural products, has been advo-

cated (172). Of all the members of the vitamin B complex, riboflavin has been found to be the most easily reducible. The diffusion current of riboflavin in a phosphate buffer of pH 7.2 is directly proportional to its concentration over the range from 5×10^{-6} to 10^{-4} M (2 to 50 p.p.m.).

For the combined determination of thiamin and riboflavin in the same sample, thiamin is adsorbed on Decalso and the filtrate is subsequently put through a column of Supersorb. The riboflavin is determined fluorometrically (173).

From biological fluids, such as urine, riboflavin can first be adsorbed on lead sulfide and then eluted with pyridine and acetic acid. Separation of riboflavin can also be accomplished directly by the addition of pyridine, anhydrous sodium sulfate, and finally, butyl alcohol to the fluid to be tested. Since butyl alcohol is miscible with pyridine but not with water, separation of riboflavin from the water can be made complete. Fluorescence of riboflavin is estimated by means of a fluorophotometer (174). Riboflavin can be quantitatively extracted from tissues by enzymatic proteolysis with pepsin (175).

Animal assay has been used less extensively. Untreated rice bran extract, containing only traces of riboflavin and apparently all other members of the vitamin B complex, has been recommended (176) as a satisfactory supplement to a diet devoid of vitamin B for use in the bioassay of riboflavin in rats.

A microbiological method (177) based on the growth response of *Lactobacillus casei* to riboflavin has yielded results which are generally in satisfactory agreement with those obtained by other methods (174, 177, 178, 179). Kemmerer (180) recommended to the Association of Official Agricultural Chemists "that both the bacteriological and fluorometric methods . . . be tentatively adopted for the determination of riboflavin in yeast and dried skim milk."

Substances which might enhance the growth-promoting activity of riboflavin in microbiological assay should be taken into account in various foodstuffs (21) and in the blood (22). Such a substance in foodstuffs, starch-like in nature, is claimed to be destroyed by taka-diastase and thus eliminated (21). The microbiological assay of urine samples with low content of riboflavin, however, shows the presence of an inhibitory factor, recognized as urea. Potential error can be avoided by adding known quantities of riboflavin, because the effect does not manifest itself when the level of riboflavin is relatively high and of urea low (181).

Distribution.—Numerous studies were completed in 1941 covering the riboflavin content of various foodstuffs (178), cereals (173), milk (173, 182, 183, 184, 185), fish (186) and fruits (187). Flavoprotein of milk remains with the raw cream on separation and passes into the buttermilk on churning. If the whole milk is pasteurized, flavoprotein is decomposed and riboflavin remains in the skim milk (188). The riboflavin content of milk does not show any significant seasonal variation (182, 185) or any relation to the content of riboflavin in the feed given the cows (183, 184). Goats fed a riboflavin-free diet continued to secrete large amounts of riboflavin in the milk (184). This independence of riboflavin secretion in the milk from the intake of riboflavin has been related to the bacterial synthesis of riboflavin in the rumen (3).

Urinary excretion of riboflavin.—The output of riboflavin in the urine reflects only the immediate dietary intake and can therefore not be considered a reliable measure of long-standing deficiency of riboflavin (189, 190). If too high doses (191) are avoided, the "saturation" test gives a fairly reliable estimate of a deficiency state with regard to riboflavin (189, 190). When 1 mg. of riboflavin (or, better, 0.016 mg. per kilogram of body weight) was given intravenously, the urinary output of riboflavin four hours after injection showed 32 to 72 per cent in normal and 81 to 93 per cent in deficient subjects retained in the body (190). Studies of riboflavin balance in ten women kept under institutional care and fed a controlled diet indicate that the daily requirement of riboflavin for an adult is approximately 3 mg. (192).

Animal experiments.—Increasing the fat ratio in a diet low in riboflavin has an aggravating effect on the production of the deficiency syndrome in rats but the deficiency can be completely corrected by addition of riboflavin to the diet (193). In severe deficiency of riboflavin, such as that observed in rats fed a high fat ration, partial paralysis of the legs is often encountered. The lesions are correlated with myelin degeneration and gliosis in the spinal cord and with degeneration of the myelin sheaths of the nerves accompanied by axis cylinder swelling and fragmentation (194).

The cutaneous lesions seen in rats suffering from riboflavin deficiency have been thoroughly studied (195). Activity of xanthine oxidase, a flavoprotein, is greatly diminished in the livers of riboflavin-deficient rats and shows a two- to five-fold increase after administration of riboflavin (196, 197). Even when deficiency of

riboflavin is far advanced in rats, no anorexia develops. Riboflavin added to the diet produces a decided effect on the economy of food utilization for synthesis of body tissues (198, 199). The phosphorylated form of riboflavin (and of thiamin) had no beneficial effect on adrenalectomized rats (200). Thus has been further disproved the theory of Laszt & Verzár (201), according to which adrenalectomized rats have, in general, lost their capacity of phosphorylation, including that of riboflavin, and in consequence are suffering from a "secondary avitaminosis." Malignant hepatoma in rats following ingestion of dimethylaminoazobenzene (butter yellow) can be prevented, to a large extent, by the combined administration of casein and riboflavin (202).

The riboflavin requirement for the growing dog is about 400 μ g. per 100 gm. of dry ration. In riboflavin-deficient dogs the blood riboflavin values show a decrease of 27 per cent from normal, the urinary excretion of riboflavin is markedly reduced and the "saturation" test is distinctly positive (203). Opacities of the cornea and neurological abnormalities develop, accompanied, as in rats (194), by myelin degeneration of the peripheral nerves and the posterior column of the spinal cord (204). In pigs, in addition to retarded growth and corneal opacities, the changes characteristic of riboflavin deficiency are scaly and ulcerated skin; pitted and ridged hoof; gray, thin, rough hair; and a terminal collapse associated with hypoglycemia (205), as previously described in dogs (206).

Clinical observations.—In extension of previous reports, it is claimed that riboflavin deficiency is exceedingly common in adults (207, 208) and that, at least in some districts, as in an area of Alabama in which deficiency diseases are endemic, it is also very widespread among infants and children (209). With regard to symptomatology nothing new has been added to the known manifestations, consisting of vascularization of the cornea, rosacea keratitis, cheilosis, seborrheic lesions about the ears and nose, glossitis, and general symptoms (207 to 215). Specific therapy with riboflavin has been found satisfactory.

The report (216) that even nonvascular keratitis benefits from treatment with riboflavin needs further confirmation. The claim (217) that in a case of Ritter's disease in a young infant, administration of riboflavin was beneficial, does not appear to be well substantiated. Furthermore, the clinical picture described better fits that of Leiner's disease (erythroderma desquamativum).

PYRIDOXIN

Methods of determination.—A successful bioassay of pyridoxin in rats depends on the use of a ration devoid of pyridoxin but complete in every other respect. As a supplement to be added to a vitamin B-free diet which would fulfill this condition, a specially prepared liver extract has been recommended (218).

The microbiological method is based on the observation that yeast, as well as certain bacteria, notably lactic acid bacteria (219), grow only in the presence of pyridoxin. *Saccharomyces cerevisiae* (Strain G.M.) has been selected as the test organism for quantitative tests (220). The values obtained are generally considerably lower than those obtained by bioassays and chemical methods. An attempt was made to explain this discrepancy by the assumption, not yet proved, that whereas the microbiological method measures pyridoxin alone, the bioassay and the chemical method jointly determine two or more closely related factors (220). It appears more probable, however, that the pyridoxin, which occurs in tissues to a large extent, in bound form, was incompletely extracted in the microbiological assay.

One colorimetric method (221) for estimation of pyridoxin, to be used also in assaying foodstuffs, is based on the azo color produced by the vitamin when it is acted upon by diazotized sulfanilic acid and *p*-nitroaniline, respectively, in an alkaline medium. The method is claimed to be highly sensitive, 5 to 10 μ g. of the vitamin being easily estimated. Interfering substances have to be removed. In a second, apparently more specific colorimetric method (222), a 2,6-dichloroquinonechloroimide is used. Inasmuch as pyridoxin will not react in the presence of a borate buffer under the conditions of the test, the vitamin may be differentiated from other substances which do react when a borate buffer is present.

By means of a rat-growth method (223), the content of pyridoxin in meat and meat products has been determined. Kidney and muscle were the richest sources, heart and liver somewhat lower, and spleen, pancreas, brain, and lung contained only about one third of the amount present in liver or muscle. Roasting and stewing caused losses of from 20 to 50 per cent. On the basis of a daily human requirement of about 2 mg. of pyridoxin, 300 to 400 gm. of fresh muscle would be necessary to supply this minimum (223).

Animal experiments.—Incorporation of dried egg white into a synthetic ration, that is free from the vitamin B complex and supple-

mented with thiamin and riboflavin, increases the incidence of acrodynia in rats (224). Gangrene and spontaneous amputation of the digits in rats, produced by the combined deficiency of pyridoxin and the "filtrate fraction," has been studied histologically (225). Combined deficiency of pyridoxin and potassium produces extensive damage of the myocardium in rats and small pigs (226).

No supplementary relationship in requirements for pyridoxin and essential fatty acids could be established and, in particular, no "sparing action" of unsaturated fatty acids on pyridoxin was observed (227). The manifestations of deficiency of pyridoxin and of essential fatty acids are somewhat similar but the two factors are not interchangeable (228). In view of the observation that in rats various combinations of the members of the vitamin B complex do not promote synthesis of fat from protein unless pyridoxin is present, it was suggested (229) that pyridoxin might be essential for the metabolism of protein.

In dogs kept for 300 days or longer on a diet free from pyridoxin, symptoms of cardiac failure (dyspnea, tachycardia, dilatation, and hypertrophy of the right side of the heart and passive congestion of the liver), together with peculiar attacks of periodical transitory weakness and prostration, were noticed (230). The neurological manifestations may be the equivalent of the epileptic fits seen in pyridoxin-deficient rats, swine, and dogs. The severe microcytic anemia which accompanied the pyridoxin deficiency in the dogs responded to pyridoxin concentrate but not to crystalline pyridoxin or to iron (230).

The growing chick requires pyridoxin in its feed, the optimal amount being between 300 and 500 $\mu\text{g.}$ per 100 gm. of the ration (231).

In ruminants the question of supply is again complicated by the fact that pyridoxin can be synthesized in the rumen and reticulum of the sheep and in the rumen of the cow. Milk from a cow fed a B-complex-deficient diet contained at least as much pyridoxin as milk from cows fed a normal ration (5).

Physiology and pharmacology.—The importance of pyridoxin as an essential nutrilit extends to higher plants. It was of distinct benefit to excised tomato roots (232).

In combination with thiamin, pyridoxin added to the diet (233) or given parenterally (234) brought about a striking reduction in the number of oocysts produced in rats infected with standardized doses of the coccidium *Eimeria nieschulzi*. It was claimed (235) that pyri-

doxin as well as yeast extract administered subcutaneously but not orally cures and prevents anemia produced in rabbits by typhoid toxin prepared from a broth culture.

Six out of seven patients with postencephalitic Parkinson's disease had a diminished urinary output of intravenously administered pyridoxin. Similar low excretion was often found in apparently normal subjects over fifty years of age without renal impairment. In patients with renal insufficiency the urinary excretion of pyridoxin was also diminished (236). Pyridoxin given *per os* (100 mg.) is followed by rapid increase of a bluish-purple fluorescence in the urine, origin of which is not yet elucidated (237).

Clinical use.—Reports on the clinical application of pyridoxin, as was the case with thiamin, showed during 1941 a more critical approach than in the past. On the whole the results obtained with pyridoxin therapy were discouraging. In progressive muscular dystrophy and atrophy, and in amyotrophic lateral sclerosis, pyridoxin given either alone or in combination with α -tocopherol has been found ineffective, regardless of whether it was administered by mouth or by a parenteral route, in small or in large doses (113, 238 to 243). Its use has been found disappointing also in Parkinsonism (112, 113, 244 to 250). Improvement, if any, was very irregular and was observed in not more than 10 to 20 per cent of the patients treated (246). If improvement occurs, it is probably due to nonspecific action of pyridoxin on muscle metabolism and not to relief of a state of deficiency (113).

It is claimed that pyridoxin was of benefit in the treatment of three cases of Sydenham's chorea (251) and of a specific type of glossitis, characterized by smooth, slightly edematous tongue with a peculiar purplish or magenta hue (208).

PANTOTHENIC ACID

Nomenclature.—R. J. Williams, who first coined the name pantothenic acid, has opened anew to discussion the question of nomenclature by his recent recommendation of a simpler term "pantothen" (252). His suggestion has yet to be accepted by the proper committees on nomenclature.

Chemistry.—Sodium *d*-pantothenate has been considered to possess certain advantages over the calcium salt for use as a primary vitamin standard (253). It has been prepared (*a*) by the fusion of

α,γ -dihydroxy- β,β -dimethylbutyramide with the sodium salt of β -alanine, (b) by the fusion of sodium α,γ -dihydroxy- β,β -dimethylbutyrate with β -alanine, or (c) by refluxing a solution of α -hydroxy- β,β -dimethyl- γ -butyrolactone and the sodium salt of β -alanine in ethanol or isopropanol (253).

The resolution of racemic pantothenic acid can be effected by means of its quinine (254, 255, 256) and cinchonidine salts (257), the *d*- and *l*- salts having different solubilities.

Methods of determination; distribution.—No chemical test is yet available for the estimation of pantothenic acid. The biological assays use either the chick growth method (258) or the growth response of bacteria, such as *Lactobacillus casei* ϵ , with varying composition of the medium (24, 259), or *Proteus morganii* (260). Complete extraction of pantothenic acid, which is a prerequisite for a successful microbiological assay, is hampered by the fact that drastic procedures, such as hydrolysis, cannot be applied without destruction of the pantothenic acid. Relying by necessity on milder methods of extraction, for example, on autolysis (24), the microbiological assays give generally lower values than are obtained by animal tests, as can be easily understood. In addition, the microbiological test organisms, *Proteus morganii* and *Lactobacillus casei* ϵ appear to respond differently to the same amount of pantothenic acid, at least when the test is carried out on blood. For instance, the content of pantothenic acid in human blood was found to be, on an average, with *Proteus morganii* 5.9 $\mu\text{g.}$ per 100 cc. (261) and with *Lactobacillus casei* ϵ 19.4 $\mu\text{g.}$ per 100 cc. (262).

The content of pantothenic acid in the blood of various animal species (dog, horse, pig, rabbit, sheep) has also been determined, with special reference to its distribution between plasma and cells (262). The concentration of pantothenic acid in the egg has been found directly proportional to the supply in the ration fed the hens (263). It is interesting to note that royal jelly is apparently the richest source of pantothenic acid. It contains about six times as much pantothenic acid as is present in yeast or liver (264). Cooking of foods does not entail any extensive destruction of pantothenic acid (265).

The foregoing data have been obtained by the microbiological method. By the more reliable biological assay on chicks sixty-eight different foods and other products of natural origin have now been analyzed (258) for content of pantothenic acid. Good sources of pantothenic acid containing more than 28 $\mu\text{g.}$ per gram of dry material are, in order of decreasing content of pantothenic acid, brewer's yeast, liver,

egg yolk, broccoli (leaves and blossom), cane molasses (blackstrap), peanut meal, and whey (258).

Animal experiments.—In rats fed a diet devoid of the vitamin B complex and supplemented with thiamin and riboflavin and with or without choline, deficiency of pyridoxin becomes the prevailing disease, cure of which by administration of pyridoxin is followed by symptoms traceable to deficiency of pantothenic acid (224). The two conditions often seem to overlap. Their distinction is perhaps best exemplified by the difference in the cutaneous lesions, which in deficiency of pantothenic acid and in the presence of pyridoxin are characterized more by generalized scaly dermatosis and alopecia than by acrodynia (224). In contrast, in mice the combined deficiency of pyridoxin and pantothenic acid gives the dominant position to the latter (266), characterized, and this is true also in the presence of pyridoxin, by suppressed growth and scaly dermatosis with widespread alopecia (266 to 270), by spinal curvature, serous exudate around the eyes, and twitch (268) or paralysis (269) of the hind legs. On histopathological examination myelin degeneration was found in the sciatic nerves and in the spinal cord (271). The suprarenal glands remained normal in mice (271), whereas in rats suffering from deficiency of pantothenic acid the common finding of hemorrhagic necrosis in the suprarenal glands and its prevention by calcium pantothenate have again been confirmed (272, 273).

In rats, daily administration of 5 μ g. of calcium pantothenate prevents hemorrhagic necrosis in the suprarenal glands. Daily doses of between 10 and 50 μ g. produce large growth increment which is proportionately less with doses between 50 and 100 μ g. With doses higher than 100 μ g. there is no significant additional growth response (273). In mice a dose of 30 μ g. of calcium pantothenate represents the daily optimum (268, 269).

For permanent cure of the cutaneous manifestations observed in rats kept on a diet devoid of pyridoxin and pantothenic acid, both these vitamins are required (267, 274, 275). As a third factor the essential fatty acids have to be taken into account (274, 275).

The syndrome of deficiency of pantothenic acid in rats can be precipitated by chronic zinc chloride poisoning and the toxic effect accordingly neutralized by proper doses of pantothenic acid (276).

The so-called "blood-caked" whiskers often found in rats that show signs of deficiency of pantothenic acid are due to the presence of coproporphyrin (277, 278), which is derived from the Harderian

gland (278). This incrustation with porphyrin in the nasal region can be produced in normal rats by partial dehydration (279). Whether this observation indicates that pantothenic acid may be involved in the regulation of water metabolism (279) or that the occurrence of "blood-caked" whiskers is caused by some unspecific disturbance of the water metabolism is still an open question.

Pantothenic acid and nutritional achromotrichia.—The beneficial effect of pantothenic acid, given in sufficient doses (> 50 μ g. daily), on the achromotrichia seen in rats kept on a routine diet free of pantothenic acid, has been confirmed by several investigators (228, 267, 273, 280, 281, 282, 283). Cure of the so-called pattern graying is practically complete in the animals treated. If any graying remains, it has only a stippled appearance (267, 280, 281). The efficacy of liver extract or rice bran extract in achromotrichia parallels their content of pantothenic acid (280). At variance with this conclusion is the statement that liver extract is more effective in the cure of achromotrichia than its content of pantothenic acid would indicate (284). Supplements of choline (285) or of cystine (286) have a synergistic effect on pantothenic acid in the therapy of achromotrichia in rats. In contrast, low intake of sodium chloride accelerates graying in rats which are fed a diet low in pantothenic acid (287). Extracts of the adrenal cortex, of the thyroid gland and of the anterior part of the pituitary gland as well as desoxycorticosterone had no effect on achromotrichia in rats kept on diets deficient in pantothenic acid (288). The effect of pantothenic acid in achromotrichia is not limited to rats but could be observed under similar conditions also in mice (267).

On a diet which contains the water-soluble vitamins, thiamin, riboflavin, nicotinic acid, pyridoxin, choline and pantothenic acid, rats attain maturity (280, 284, 289, 290) and even bear litters (280, 289, 290). In mice the addition of some other unknown factors, different from inositol and *p*-aminobenzoic acid but present in liver extract, seems to be necessary to support normal development (268, 291). Pantothenic acid can be synthesized to a large extent in the rumen of cows and in consequence often more pantothenic acid is excreted by way of the milk than can be accounted for in the ration fed (7). The increased content of pantothenic acid in eggs, caused by supplementing the ration of hens with pantothenic acid (263) or by direct injection into the egg before incubation, brought about better embryo survival time, thus indicating increased hatchability (292, 293). Monkeys (*Macacus mulatta*) maintained on a diet deficient in pantothenic

acid showed severe oral lesions which were accompanied by an increase in the fusospirochetal flora (294).

Urinary and fecal excretion.—The urinary excretion of pantothenic acid, after its administration orally or parenterally, has been studied in man and in rabbits (295, 296). In contrast to the urinary output of pantothenic acid, its fecal excretion in rats is independent of intake and is probably determined by the activity of the intestinal flora (273).

Pharmacology and toxicology.—In analogy to the relationship of sulfanilamide and *p*-aminobenzoic acid, it has been found that the growth of *Lactobacillus arabinosus* and yeast can be inhibited by the sulfonic acid analogue of pantothenic acid, *N*-(α,γ -dihydroxy- β,β -dimethylbutyryl) taurine, and that the inhibition can be reversed by addition of large amounts of calcium pantothenate (297, 298).

In short term as well as in longer experiments, pantothenic acid has shown only very slight toxicity. The L.D. 50 of calcium pantothenate following its subcutaneous injection is 2.7 gm. per kilogram of body weight in mice and 3.4 gm. per kilogram in rats (299). Daily administration of calcium pantothenate over a period of six months to monkeys (1 gm. per animal), to dogs (50 mg. per kilogram) and to rats (50 and 200 mg. per rat) failed to produce any untoward effects or pathological changes (299).

CHOLINE

During the year 1941 a great deal of progress was made in the elucidation of the role played by choline as a dietary essential, especially in perosis, in hemorrhagic cortical necrosis of the kidney, and in hepatic injury characterized by focal or diffuse necrosis and cirrhosis.

Perosis.—Both manganese and choline are necessary for growth and for the prevention of perosis in turkeys and in chicks (2, 300 to 304). Perosis did not develop in chicks, in contrast to turkeys, in the absence of choline, unless gelatin or creatine was added to the ration (301). Creatine acts, perhaps, by intensifying the muscle tonus in the legs (301). Choline prevents development of perosis at a level of 0.1 gm. per 100 gm. of the ration; lower levels are only partially effective (301). Choline can be replaced in the diet by lecithin but not by inositol or methionine (2). The inefficacy of methionine is especially noteworthy, methionine being otherwise a completely satisfactory substitute for choline, as, for example, in the prevention of

cortical necrosis of the kidney (305) and of hepatic injury in rats (306).

Bone phosphatase values were normal in chicks suffering from perosis due to choline deficiency (303), in contrast to the lower values in chicks with perosis caused by deficiency of manganese (307).

It was recently claimed that in addition to choline there exist at least two other organic nutrients concerned in the prevention of perosis. One of these substances is biotin, and will be discussed later. The other is not identical with any of the recognized vitamins and is present in aqueous liver extract from which it can be adsorbed on fuller's earth and subsequently eluted with 0.2 *N* ammonia (308).

Hemorrhagic cortical necrosis of the kidney.—Very high levels of casein (30 per cent or more) have been found necessary to supply sufficient methionine to permit omission at the same time of a choline supplement in the diet of young rats 21 to 26 days old (309). On diets containing less casein and no choline, hemorrhagic cortical necrosis of the kidney occurs regularly in rats and can be prevented by 1 to 2 mg. of choline chloride daily (309). Older rats maintained on a diet containing 24 per cent of casein apparently have no need of choline (281). Hemorrhagic cortical necrosis is aggravated by cystine (305), by peanut protein (310) and by cholesterol (311). The effect of cholesterol is prevented by addition of choline in short term experiments (8 days) although not completely if the experiment is extended for 30 days (311). Betaine is less effective than choline in the prevention of hemorrhagic degeneration (312), which is also less severe when the consumption of food is restricted (311).

Hepatic injury (necrosis and cirrhosis).—In rats kept on a diet low in casein, free from choline and preferably but not necessarily high in fat, and supplemented with thiamin, riboflavin, pyridoxin and pantothenic acid, hepatic injury (necrosis and cirrhosis) became a regular complication (306, 313, 314, 315, 316). Supplements of cystine had an aggravating effect on the production of cirrhosis, whereas a preventive influence was exerted by a higher casein ratio and addition of methionine and choline, the latter more noticeably (313) when in combination with cystine (306, 313, 314, 315).

Experiments on hepatic injury in rats following the ingestion of "butter yellow" have shown that the oral administration of cystine and choline simultaneously is beneficial but that cystine alone aggravates the hepatic injury, which remains uninfluenced by the feeding of choline alone (317).

Whether or not the satisfactory results reported (318) in the treatment of cirrhosis of the liver in man by a "highly nutritious diet," richly supplemented with the vitamin B complex, are really due to a higher intake of protein and of choline, needs further special study.

Young puppies kept on a synthetic diet containing only 19 per cent of casein require a supplement of choline for normal growth (319) just as do chicks and young rats.

The relationship of methionine and choline has been explained by the utilization of the methyl group of methionine in the biological synthesis of choline (320). Betaine may fulfill the same function (321, 322). These considerations and experimental results, however, leave unanswered the following questions: why is the presence of cystine necessary to obtain the full effect of choline? and, why can methionine replace cystine plus choline?

BIOTIN

Identification of the curative factor for egg white injury (vitamin H) with biotin and coenzyme R, as announced and definitely established in 1940, opened the way for intensive chemical and biological research. At the present time it is still in the center of general interest.

Biotin, which was known in the past only as an exceedingly potent growth promoting factor for yeast and bacteria, such as *Rhizobium*, became a member of the vitamin B complex after its role in the egg white injury of animals was elucidated. The terms vitamin H and coenzyme R have been abandoned and the hitherto independent pathways of study merged in research on biotin.

Chemistry.—The year 1941 brought great progress in the analysis of the molecular structure of biotin. In crystalline form the methyl ester of biotin was first obtained in 1936 in Kögl's laboratory from egg yolk (323) and its empirical formula was reported in 1938 (324). From a liver concentrate (26) the same compound was obtained in the laboratory of du Vigneaud in 1940 (325) by means of a procedure, details of which were described later (326). After repeated crystallization and sublimation the compound possessed constant biological activity and a constant melting point of 166° to 167° (326). The melting point of the compound prepared from egg yolk was originally given as 148° (323), but was later corrected to about 161.5° (327). The biological potency of the compound obtained from liver was found, by means of the yeast growth method as well as by the curative method in assays on rats suffering from egg white injury, to be consistently

27,000 (± 10 per cent) vitamin H units per milligram (325, 326). With this high activity biotin represents biologically the most potent member of the vitamin B complex and is in general physiologically one of the most active chemical substances known.

The analytical data concerning the methyl ester of biotin prepared from liver agree very closely with the empirical formula $C_{11}H_{18}O_3N_2S$ (326), which is identical with that found for the compound isolated from egg yolk (324). Free biotin, obtained from the ester, melted at 230° to 232° and its composition $C_{10}H_{16}O_3N_2S$ is in agreement with the results of analyses of the ester (328). Both the methyl ester and free biotin are optically active, with a specific rotation of $[\alpha]_D^{22} = +57^\circ$ for a 1 per cent solution of the ester in chloroform (326) and $[\alpha]_D^{22} = +92^\circ$ for a 0.3 per cent solution of free biotin in 0.1 N NaOH (328).

To treatment with acid or alkali, pure biotin does not appear to be as stable as biotin in impure concentrates. Furthermore, pure biotin is easily destroyed by hydrogen peroxide, by peroxide-containing ether or aqueous solutions of bromine and by nitrous acid, but it does not react with ninhydrin (329).

In several publications, based on independent investigations, du Vigneaud and Kögl and their respective collaborators have made important contributions to the elucidation of the structure of biotin (326, 330 to 334). Both groups are in agreement that biotin is a monocarboxylic acid with a double ring system. One ring represents a cyclic urea structure and the other ring contains sulfur as a part of a ring in a thio ether linkage (327, 330, 331, 333). The urea structure was confirmed by the resynthesis (332) of biotin from the diamino-carboxylic acid obtained by treatment of biotin at 140° with barium hydroxide (331) and phosgene. Recently adipic acid has been isolated from the oxidation of the diaminocarboxylic acid derived from biotin (333) and then it was shown that one of the carboxyl groups of the adipic acid was the original carboxyl group of biotin (334).

All these experimental results and pertinent considerations greatly reduce the number of possible structures that may be ascribed to biotin. It is hoped that final solution of this problem will be achieved shortly.

Methods of assay.—Identification of the curative factor for egg white injury with biotin eliminated the necessity of animal assay for the estimation of biotin and allowed substitution for it of the simpler and quicker microbiological method. As test organisms yeast (335), *Clostridium butylicum* (27) and *Rhizobium trifolii* (336) have been recommended.

For complete extraction of biotin, commonly present in bound form in foodstuffs and similar products, acid hydrolysis is, in general, the most suitable procedure (25, 26, 27, 335, 336).

Distribution.—Earlier data on the distribution of biotin (vitamin H), obtained by animal assays, have been widely extended by the more recent microbiological methods (25, 27, 335, 336).

Particular attention has been paid to the content of biotin in animal tissues (92, 335, 336). Especially rich in biotin (92, 336) are liver, kidney, and also pancreas, which latter, in contrast to liver and kidney, is a rather poor source of other vitamins. With regard to variations during the developmental process, it is noteworthy that in the rat embryo the skin, skeletal muscle, brain, and lung contain amounts of biotin greatly in excess of those found in the corresponding adult organs (336). Heart, kidney, and liver, however, of the embryo contain less biotin than do these organs in the adult, which are notably rich in biotin, perhaps owing to the role of these organs as storage depots in the adult (336). The possible relationship of biotin to the fundamental process of growth is illustrated by the observation that the biotin content of tumors deviates sharply from the normal adult values, and in the same direction as that of the corresponding embryo tissues (336).

Egg white injury.—The induced nature of egg white injury was first suggested by experiments in which feces obtained from rats suffering from egg white injury were fed to like animals. The feces were found to be nonpotent but were highly active after steaming. It was assumed that some constituent of egg white combines with and holds in nonabsorbable form the curative factor which originated either from the diet alone or was excreted into the digestive tract. The nonabsorption of the curative factor would then be responsible for the "egg white injury" (337).

The results of these animal experiments were confirmed and given further support by investigations in which the inactivation of biotin by egg white was demonstrated on yeast cells (338). The specific constituent of egg white responsible for this effect has now been concentrated and purified (338, 339). This protein-like substance, termed avidin (339), forms with biotin a fairly stable compound from which biotin can be released by steaming, similar to the behavior of the avidin-biotin complex (AB) in the feces of rats fed a diet containing raw egg white (337, 340).

Avidin can replace raw egg white as the "toxic" factor in the diet used to produce egg white injury in rats, but only when it is given

orally (341). In contrast to this, parenteral administration of avidin preparations has a distinctly curative effect on egg white injury (340). This is explained by liberation of the biotin consistently present in avidin concentrates. The mechanism of the splitting of the avidin-biotin complex (AB) in a parenteral medium has not yet been explained. It is certainly at variance with its stability in the intestinal tract.

The only source of avidin so far known is egg white. It has been postulated that different bacteria produce avidin-like substances which through temporary inactivation of biotin are perhaps responsible for the recession of cancerous growth in man, reported in some instances after infectious diseases. This hypothesis needs further experimental verification (342).

Biotin deficiency in animals.—In the last analysis, egg white injury is induced biotin deficiency. In rats its first and apparently constant sign was claimed to be a "spectacle eye condition," which can be cured by biotin (343). Graying of the fur and its return to normal color after administration of biotin was also observed in mice fed a diet containing a large proportion of egg white (267).

Whereas no reports are known to the effect that biotin deficiency would occur in Mammalia in the absence of egg white in the diet, it can be produced in poultry, as in chicks (344, 345) and in turkey poults (346), by a diet that is devoid of biotin and contains no egg white. It is indistinguishable from that which occurs in poultry kept on an egg white diet, and both forms can be cured by treatment with biotin.

In biotin deficient chicks, perosis also was seen (4, 347). The symptoms differed, however, somewhat in appearance (302) from those caused by deficiency of choline or manganese.

Biotin and microorganisms.—Not only is biotin an indispensable nutriment for yeast, *Clostridium*, *Rhizobium*, and for a further large number of low plant cells (348, 349), including bacteria (350, 351), but some microorganisms are also known to synthesize biotin (352, 353), in many instances in unusually large quantities (354).

Biotin and liver fat.—Impure solutions of biotin, such as those prepared from a liver extract, or pure biotin caused a distinct rise in the content of fat, especially of cholesterol, in the livers of rats (355, 356, 357, 358). This biotin type of fat liver remained uninfluenced by administration of choline (355, 356, 357, 358) but it could be prevented by feeding lipocaic, egg white, or inositol (358).

OTHER VITAMIN B FACTORS

The successful correlation of essential nutrilites for microorganisms with animal nutrition, as has been noted in many instances in this review, from nicotinic acid and pantothenic acid to biotin, has facilitated a tendency to generalize. The best evidence of the vitamin character of such substances as inositol, *p*-aminobenzoic acid, and "folic acid" lies in the fact that they are known as nutrilites for microorganisms.

Inositol.—Admittedly, the incorporation of inositol in the vitamin B complex was based first on the results of animal experiments. Inositol was isolated by Woolley (359) from liver extract as the curative factor for alopecia which can be produced in mice kept on a synthetic diet containing thiamin, riboflavin, pantothenic acid, pyridoxin, and choline (359). Woolley himself stated (360, 361) that spontaneous cure of the alopecia might occur in spite of the absence of inositol, provided that pantothenic acid was given. In other investigations pantothenic acid seemed to replace inositol completely (268, 270), even when the diet originally employed by Woolley was used (362), and alopecia was never seen although no inositol was administered. It has been claimed (363) that addition of *p*-aminobenzoic acid to a diet containing all other members of the vitamin B complex brings about loss of hair in mice, which can be cured by addition of inositol. The experimental technique used in these latter investigations differed, however, from the original procedure followed by Woolley and needs further confirmation.

On the whole, the facts presented do not, at the present time, warrant the identification of inositol as a primary and really essential vitamin; they rather favor the assumption that it is a supporting and at least not always specific dietary constituent. This tentative conclusion is not disproved by observations made on animals other than the mouse concerning the role of inositol in nutrition.

The "spectacled eye condition" described in rats deficient in inositol (364) has not been seen by other authors, who were able to raise rats to maturity on a diet containing, as far as the vitamin B complex is concerned, only thiamin, riboflavin, pyridoxin, pantothenic acid, nicotinic acid, and choline (280, 284, 289, 290, 362). Inasmuch as rats may survive for three generations on this diet free from inositol (280, 289, 290), the slight beneficial effect of supplements of inositol on lactation (365, 366) can equally be regarded as not conclusive.

In chicks fed a synthetic diet deficient in inositol, no pathological symptoms other than slight retardation of growth have been observed (367). As to the lipotropic activity of inositol (358, 368) on the "biotin type" of fat liver (355, 356, 357, 358), this action can equally well be exerted by egg white or by lipocaic (358).

If in subsequent studies the vitamin character of inositol can be firmly established, the methods of its determination will gain added interest. They are based on the growth response of yeast cells (*Saccharomyces cerevisiae*) in a special medium, and are reported to be satisfactory (369, 370).

p-Aminobenzoic acid.—Interest in *p*-aminobenzoic acid as a possible member of the vitamin B complex has been stimulated by the discovery which assigned to *p*-aminobenzoic acid a specific neutralizing power on the bacteriostatic effect of sulfanilamide and related drugs (371). This antagonism to the inhibitory action of sulfa drugs applies not only in the case of bacteria (372, 373, 374) but extends also to dermatophyte fungus (375), virus (376), and even to autotrophic plants (377). It was explained (371) in general terms by the competition between sulfanilamide and *p*-aminobenzoic acid in some essential enzymatic reaction in the cells. As an example of such a reaction attention has been called to the experimental fact that the catalytic oxidation of *p*-aminobenzoic acid by peroxidase is inhibited by sulfanilamide (378).

After *p*-aminobenzoic acid was isolated from yeast cells (374, 379, 380), its essential nature for cell life became more probable. Colorimetric analysis indicated that 0.5 mg. of *p*-aminobenzoic acid, presumably as part of a peptide link, was present in 100 gm. of fresh yeast. About 60 per cent of this was recovered in crystalline form and identified chemically (380).

Among higher forms of animal life, a slight promotion of growth in chicks (381) and, in particular, anti-gray hair activity in rats (381) and mice (363, 382) have been attributed to *p*-aminobenzoic acid. Graying of the fur was reported in rats and mice fed a synthetic diet supplemented with thiamin, riboflavin, pyridoxin, choline and even with sufficient amounts of pantothenic acid (363, 381). It was also produced in mice fed hydroquinone (382). More recently the claim has been put forward that *p*-aminobenzoic acid has a positive effect on the pigmentation of hair even in man (383). These experimental and clinical observations have not yet been confirmed. As stated in connection with the anti-gray hair activity of pantothenic acid (267,

273, 280, 281) the great majority of investigators have been unable to produce severe "pattern graying" in rats fed a vitamin B-free diet supplemented with all the known members of the vitamin B complex including pantothenic acid; the addition to the diet of *p*-aminobenzoic acid had no effect (280, 362, 384). Furthermore, the relatively very high doses of *p*-aminobenzoic acid used in the animal experiments should be pointed out, namely, 3 mg. (384); only about 0.5 mg. of *p*-aminobenzoic acid is contained in 100 gm. of fresh yeast (380). Of course, it is possible that more potent derivatives of *p*-aminobenzoic acid are present in yeast, such as *p*-aminophenylacetic acid, which is ten times more potent than *p*-aminobenzoic acid (374), but proof of this assumption is still to be adduced.

With regard to the role of *p*-aminobenzoic acid as a "vitamin" required for lactation in rats (365, 366), the same comment is in order that was made in connection with inositol, namely, that three generations of rats were raised on a synthetic diet that contained all known members of the vitamin B complex except inositol and *p*-aminobenzoic acid (280, 289, 290). Emphasis should be placed also on the danger entailed by a high level of *p*-aminobenzoic acid in the blood in the case of an infection which would be otherwise easily amenable to treatment with sulfa drugs.

In another group of experiments made on rats, the neutralizing effect of *p*-aminobenzoic acid on sulfa drugs was investigated. Sulfaguanidine is absorbed from the intestine only to a very small extent and exerts its effect mainly on the intestinal flora; thus the production of vitamin-like substances by the intestinal bacteria would be inhibited. In consequence, suppression of growth in rats treated with sulfaguanidine has been correlated with such a mechanism. Liver extract, yeast, and *p*-aminobenzoic acid neutralize the effect of sulfaguanidine and allow resumption of growth (11, 12). Changes in the thyroid glands of the same animals remained uninfluenced, however, by administration of yeast or *p*-aminobenzoic acid (12). It is hoped that the results of this line of research, which should be extended also to other sulfa drugs, will shed light on the action of the sulfa drugs and in particular on the role of the intestinal flora as a source of essential nutrilites.

"Folic acid."—In studying the requirement of some lactic acid bacteria, it has been found that extract of solubilized liver contains an essential factor which can be adsorbed on norite and then eluted (385). It has been claimed that after purification the product has

the properties of a nucleotide (386) but in later experiments it became apparent that the activity does not depend on the presence of phosphorus (10, 387). The compound obtained in nearly pure form is free also from sulfur, is acidic in character (10, 387) and has a molecular weight of about 500 (10). Its destruction with nitrous acid and inactivation on acetylation or benzylation suggest the presence of an amino group (387). With *Streptococcus lactis* R. as test organism (10, 388) assays have shown that liver, kidney, mushroom, yeast, and especially green leaves and grass contain the compound in abundance. It has therefore been designated "folic" (*folium*, leaf) acid (10).

The role of "folic acid" in the nutrition of higher animals requires further study. Data obtained thus far show that rats kept on a diet free from "folic acid" manifest no (387) or insignificant (10) growth response to a supplement of "folic acid," while in chicks the response seems, perhaps, to be significant (389). The question of the synthesis of "folic acid" in the intestine needs also to be considered (10).

VITAMIN B COMPLEX

The close association of the members of the vitamin B complex in animal tissues, in foodstuffs, and in other products of natural origin makes it advisable or in some instances even necessary to consider the complex *in toto* instead of in its separate parts. For clinical and experimental problems this approach often represents only the first step to subsequent detailed analysis, whereas in dietary problems consideration of the whole B complex often is the preferable mode of attack. Clinical problems, such as cutaneous manifestations of the seborrheic type and special intestinal disturbances, appear to have some connection with the vitamin B complex. The question raised with regard to lesions of the skin was whether or not the so-called seborrheic diathesis is to a large extent dependent on faulty nutrition (390). Beneficial effects of liver extract and of yeast in cases of seborrheic dermatosis have been reported (391, 392). There still remains to be determined which cases are apt to react to treatment of this kind.

The intestinal syndrome, claimed to be due to deficiency of the vitamin B complex, is characterized by malnutrition, flat dextrose tolerance curves, and a peculiar "deficiency pattern" of the small intestine demonstrable by roentgenographic examination (393, 394, 395). The "deficiency pattern" interferes with absorption and may

therefore aggravate the deficiency disease. Celiac disease (396), ulcerative colitis, and steatorrhea (393) belong to this group, but the syndrome may be present without serious outward signs.

In animal experiments it has been shown that crystalline estrogen or androgen is inactivated in the liver but that this inactivation does not take place in rats kept on a diet devoid of the vitamin B complex (397). Further studies are needed in order to identify the member of the B complex responsible for this phenomenon.

Fortification of food, especially enrichment of bread, represents a special, widely discussed problem in which the vitamin B complex is involved (31, 111, 398, 399). If experimental proof were needed, it was presented (400, 401) to show that reinforcement of white flour with thiamin alone does not make its nutritive value equal to that of whole meal. Milling at least to 85 per cent extraction or addition of thiamin, riboflavin and nicotinic acid (and calcium with iron) or, finally, addition of "high vitamin" yeast was recommended (31, 398, 399, 402, 403). Suggested standards for the supplements of vitamins per pound of bread are thiamin 1 to 2 mg., riboflavin 0.8 to 1.6 mg. and nicotinic acid 4 to 8 mg. (399). Addition of yeast or of the B vitamins improves the biological value of the bread protein (404). As a general supplement to the ordinary diet and as an adequate source of the vitamin B complex, especially in districts where endemic vitamin deficiency diseases prevail, a mixture of yeast (25 per cent), peanut butter (67 per cent), and peanut oil (8 per cent) has been advocated (405).

ASCORBIC ACID

There appeared in 1941 the usual large number of publications dealing with ascorbic acid, but no outstanding new facts were recorded.

Chemical determination.—In the few reports published during 1941 on the use of 2,6-dichlorophenolindophenol for the chemical estimation of ascorbic acid and dehydroascorbic acid, the elimination of interfering influences or substances, such as oxidative changes or catalysts, was especially stressed (406, 407). Oxidation of ascorbic acid by the oxygen set free from oxyhemoglobin represents a possible source of error when whole blood is deproteinized with metaphosphoric acid and can be avoided by reduction of oxyhemoglobin by alternate evacuation and treatment with carbon dioxide under pressure before precipitation with metaphosphoric acid (408).

Ascorbic acid in foodstuffs and in food products.—Where citrus fruits have become scarce owing to war conditions, research has been started to find other sources of ascorbic acid. An excellent source has been found in guava (*Psidium guajava*), either fresh, canned, or dried (409). Good sources have been discovered in wild fruits, such as elderberries and wild rose hips (410), mountain plants, especially when grown in an altitude above 2400 m. (411), and to a less extent in banana (412). Fresh leaves of tea contain about three times as much ascorbic acid as orange juice. This high antiscorbutic activity is lost during processing (413), probably because of the action of ascorbic acid oxidase, a common constituent of plant cells (414). In addition to this oxidative, heat-labile enzyme, plants contain a protective factor which is thermostable and dialyzable (414).

Losses of ascorbic acid in foodstuffs during cooking and canning are due mainly to oxidation in the presence of oxygen and catalysts, and not to heat (415). Such losses may be considerable. For instance, cooking brought about a reduction in the content of ascorbic acid in potatoes of between 27 and 55 per cent and in cabbage of between 46 and 67 per cent (416). Storage may also entail losses. In potatoes the content of vitamin C fell from 0.17 mg. per gram in the fall to 0.11 mg. per gram in the spring (416). Comparative analysis of fresh and canned juice of lemon, orange, or grapefruit shows that canning under proper conditions entails only minor losses of ascorbic acid (417). For commercial preservation of vitamin C in frozen vegetables the temperature should be -18° or lower (418). Blanching with steam and rapid drying favor the retention of ascorbic acid in dehydrated vegetables. Dehydration is superior to sun drying from the standpoint of retention of vitamin C (419).

In growing tomato plants exposure to sunlight increases the content of ascorbic acid in the fruit (420). Manganese also appears to be a factor in the formation of ascorbic acid, as illustrated again in the tomato (421).

The presence of oxygen and copper and exposure to sunlight are the most important factors in the destruction of ascorbic acid in milk (422, 423). Sunlight acts through the riboflavin of the milk as photosensitizer (188). Deaeration, which is a relatively cheap commercial process costing 3 to 4 cents per 1,000 pounds of milk, and the exclusion of copper and sunlight aid greatly in preserving the ascorbic acid in milk (422).

Ascorbic acid in blood and urine; the saturation tests.—The buffy

layer of centrifuged oxalated blood, consisting mainly of leucocytes and platelets, contains a reducing substance which in chemical reaction fulfills the commonly used criteria for ascorbic acid. Its concentration, both in human beings and in guinea pigs, depends on the supply of ascorbic acid in the diet and provides a good index to significant deficiency of ascorbic acid (424). It is more reliable (425, 426) than the plasma values, which react almost instantaneously to reduction in the intake of ascorbic acid. In a human volunteer who went on a diet free from ascorbic acid, scurvy developed at the end of five months. The ascorbic acid level of the blood plasma fell to zero in 42 days and that of the buffy layer in 122 days. Thus low plasma values existed at a time when the experimental subject was in no immediate danger of clinical scurvy (426). The normal level for ascorbic acid in human blood plasma is 0.7 mg. per 100 cc. and in the buffy layer, 25 to 38 mg. per 100 gm. of white blood cells (425). Although low plasma concentrations of ascorbic acid should not necessarily be attributed to clinical scurvy, they indicate, as a rule, low dietary intake of ascorbic acid. In healthy human subjects, twelve to thirty-five years of age, the concentration of ascorbic acid in blood plasma is around 1 mg. per 100 cc., provided that the dietary intake of ascorbic acid is around 1.7 to 1.9 mg. per kilogram of body weight (427). To maintain an ascorbic acid level of 0.7 mg. per 100 cc. of plasma an adult requires 75 mg. of ascorbic acid daily (425). In a large proportion of apparently healthy children (428) who live in such different parts of the United States as Maine (416) and Tennessee (429), levels of ascorbic acid in the blood plasma have been found which indicate unsatisfactory (0.3 to 0.7 mg. of ascorbic acid per 100 cc. of plasma) or even seriously low intake of vitamin C (ascorbic acid level under 0.3 mg. per 100 cc. of plasma) (416, 428, 429, 430, 431). Higher intake raises the level of ascorbic acid in plasma (431).

The concentration of ascorbic acid is considerably higher in the plasma of the newborn infant (average 1.16 mg. per 100 cc.) than in the plasma of the mother (average 0.42 mg. per 100 cc.). In the first two weeks of life the plasma values for ascorbic acid in infants vary with the amount of ascorbic acid in the diet (432).

The status of ascorbic acid can be better assessed by saturation tests than by the single determination of ascorbic acid in plasma. In saturation tests the rise of the ascorbic acid level in the blood is determined after injection (433) or in the urine after ingestion (434) of

large doses of ascorbic acid. The higher the rise, the better is the organism supplied with ascorbic acid. Both tests work satisfactorily (433, 434).

With the help of saturation tests of this kind and of the recorded blood plasma values of ascorbic acid, various conditions have been analyzed with respect to possible deficiency of ascorbic acid. Investigators have found varying proportions of school children with a severe degree of undersaturation (430, 435, 436). Once saturation is reached, school children need a daily intake of 31 mg. to maintain it (436). Deficiency of ascorbic acid is claimed to be widespread in the aged. In a series of twenty-five so-called normal aged persons only two showed a satisfactory saturation test (437). Patients with tuberculosis (435, 438) and bronchial asthma (439) have a higher requirement of ascorbic acid than normal persons, which is particularly evident in the more advanced cases of tuberculosis. In gastric diseases deficiency of ascorbic acid is equally common (440). Here due account should be taken of the fact that, owing to failure of absorption (441) and perhaps because of direct excretion (442), ascorbic acid may be lost with the feces. In the healing of wounds in man general nutritional conditions play an important part. Delayed healing and disruption of the wound may be the result of ascorbic acid deficiency (426, 443, 444, 445).

Animal experiments.—Guinea pigs with experimental scurvy have an increased basal metabolic rate and show changes in the thyroid gland in the direction of hyperactivity (446).

The effect of ascorbic acid deficiency on two hydrolytic enzymes (liver esterase and phosphatase of the intestinal mucosa and kidney cortex) and two respiratory enzymes (succinic dehydrogenase and cytochrome oxidase) in guinea pigs was studied. Without claiming specificity, a definite decrease of esterase activity in the liver, a marked drop in succinic dehydrogenase activity and only a moderate decrease in cytochrome oxidase activity in skeletal and cardiac muscle was found; the phosphatase activity remained practically unchanged (447).

During advanced scurvy in guinea pigs gastric secretion under histamine stimulation is distinctly reduced. Owing to increased capillary fragility, gastric ulcers may develop in consequence of a breakdown in the capillaries of the gastric mucosa and submucosa (448). Under the demands of heavy egg production in hens kept on a diet low in ascorbic acid, there may develop a true deficiency of vitamin C,

characterized by "leg weakness," inanition, and depression of egg production, all curable by injection of ascorbic acid (449). The level of ascorbic acid in blood plasma of cows shows only slight variation and remains unchanged even after ingestion of 100 gm. of ascorbic acid, but it can be raised by injection of much smaller doses (450). This observation is explained by the fact that ascorbic acid is destroyed in the rumen of the cow. Ascorbic acid is intimately associated with early phases of the reproductive process in the cow, and when it is given by injection it has a beneficial effect in the treatment of certain types of sterility in that animal (451).

Physiology.—Lemberg and his collaborators (452, 453) studied extensively the products found in the course of prolonged coupled oxidation of hemoglobin and ascorbic acid, such as choleglobin and cholehemochromogen. They point out (454) that the breakdown of hemoglobin to bile pigments with the removal of iron may take place with ascorbic acid at physiological values of pH. Reduction of methemoglobin by ascorbic acid has also been investigated (455).

Premature infants, in contrast to full term infants, who received diets of relatively high protein content (5 gm. or more per kilogram of body weight per day), showed as early as the sixth day of life a spontaneous defect of the metabolism of tyrosine and phenylalanine. This defect persisted as long as ascorbic acid was withheld but was relieved by the administration of ascorbic acid. The defect is manifested by the excretion of 1-*p*-hydroxyphenyllactic and *p*-hydroxyphenylpyruvic acids in the urine, compounds which were chemically identified (456, 457).

Similar disturbance of the metabolism of phenylalanine and tyrosine was found in scorbutic guinea pigs, with corresponding beneficial effect of administration of ascorbic acid (458).

Pharmacology.—The minimum stimulating dose of histamine is one thousand times greater for muscle preparations obtained from scorbutic guinea pigs than for preparations from normal animals (459). Intravenous injection of ascorbic acid prolongs the life of cats after they have lost 50 per cent of their original blood volume by hemorrhage (460).

Sodium diphenylhydantoinate (dilantin), a drug recently introduced in the treatment of epilepsy, produces in guinea pigs (461) and even in rats (462) a rapid and progressive fall in the ascorbic acid level of the blood (461) and of the tissues, and an increase in the urinary excretion (462), with return to normal levels after with-

drawal of the drug (461). No similar effect was noticed on the ascorbic acid content of blood plasma and urine after administration of 180 mg. of phenobarbital daily to human subjects (463). The narcotic effect of pentobarbital sodium (nembutal) was markedly prolonged in guinea pigs fed a diet free from ascorbic acid (464, 465).

Ascorbic acid may play a role in the inactivation of amphetamine (benzedrine). With massive doses (200 to 400 mg.) of ascorbic acid in dogs the urinary excretion of the drug decreases. The inactivation could also be demonstrated *in vitro* (466).

Arsenical sensitivity and ascorbic acid.—Ascorbic acid is effective in the cure of arsenical sensitivity following treatment with neoarsphenamine or mapharsen, especially in arsenical dermatitis (467, 468, 469, 470). Hypovitaminosis C appears to promote the manifestations of this sensitivity (467, 469). In the majority of hypersensitive patients typical cutaneous reactions, as shown by patch tests, could be prevented by the application of a sufficient amount of ascorbic acid (470). By maintenance of a high level of ascorbic acid in the blood, even sensitive patients should be able to tolerate administration of arsenical drugs (469, 470). Ascorbic acid and arsenicals must not be given in a mixture, however, but rather given separately (467) because the stabilizing effect of ascorbic acid on solutions of arsenical drugs (470) is not a constant phenomenon. Sodium neoarsphenamine is precipitated by ascorbic acid (467).

Use of ascorbic acid in conditions other than scurvy.—Contradictory reports appeared concerning the influence of ascorbic acid on diabetes and on the insulin requirement in diabetes (446, 471). It is claimed (472) that muscular pain brought about by intense military training subsided under the influence of ascorbic acid (200 mg. daily) and that the performance of the untreated controls was decidedly worse than that of the treated subjects. A combination of thiamin (10 mg.) and ascorbic acid (50 mg.) administered daily is reported to have relieved the nausea and vomiting of radiation sickness (125). Routine administration of 50 mg. of ascorbic acid daily appears to protect workmen against the usual effects of chronic lead absorption and promotes the excretion of the metal (473).

VITAMIN P

The experimental basis for the recognition of citrin (a mixture of hesperidin and eriodictyol glucoside) as a vitamin still remained uncertain in 1941. As a new test animal, the rat was recommended.

It was claimed (474) that in rats kept on a scorbutogenic diet the capillary resistance diminished in from five to six weeks without development of signs of scurvy; subcutaneous injection of 3 to 4 mg. of citrin per day restored capillary resistance to normal in from ten to fourteen days.

As in past years, favorable results after treatment with vitamin P were reported in clinical studies involving scattered observations, which, however, were not well controlled. This group includes patients in whom hemorrhagic diathesis developed after neoarsphenamine therapy (475, 476) and after measles (477), cases of thrombocytopenic purpura (478) and other subjects who showed increased capillary fragility (479, 480).

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FAT-SOLUBLE VITAMINS

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After a period of notable advances which have been rapidly confirmed, the study of vitamin A has entered upon a difficult phase in which careful experiments yield apparently contradictory results. It is now realised that the subject is very complicated and the spurious clarity which was induced by too simple a picture has gone altogether. Discrepancies and anomalies are being faced, however, and rapid progress towards a more significant interpretation is to be expected.

A possible new member of the Vitamin A and A₂ group has been suggested in "subvitamin A" (1). The compound is said to be present in liver oils from several species of fish, and to be characterised by an ultraviolet absorption maximum at 310 mμ. It exhibits a maximum at 620 mμ in the antimony trichloride colour test, and on cyclisation yields a product with an absorption spectrum slightly different from that of cyclised vitamin A and A₂. The maximum at 310 mμ has often been recorded as an inflexion in the curve for vitamin A ($\lambda_{\text{max}} = 325$ mμ) but the idea of a "subvitamin A" is new. Detailed studies of carotenoids in palm oil (2) and watermelon (3) have been made.

VITAMIN A

Determination of vitamin A activity.—Discussions (4) arising out of collaborative investigations (5 to 9) have now reached a point such that the whole question of vitamin A activity may profitably be reviewed briefly. The subject is complicated by the existence of vitamin A activity due to preformed vitamin (C₂₀H₂₉OH or its esters) and to carotenoid provitamins converted *in vivo* to vitamin A.

The International Unit was intended to apply to both aspects and was defined in 1931 (10) as the activity of 1 μg. of "carotene." The discovery of carotene isomers α-, β-, and γ- soon rendered the original standard preparation obsolescent, and in 1934 (11) the International Unit was redefined as the activity of 0.6 μg. of pure β-carotene. This redefinition preserved continuity in the International Unit within the limits of error governing the direct comparison (by bioassays) of the original standard "carotene" with the purest β-carotene.

tene then available. Insofar as analytical chemistry is concerned, the activity of β -carotene is 1.66×10^6 I.U. per gm. The physical properties of β -carotene, particularly molecular extinction coefficients at absorption maxima in specified solvents, are fairly well agreed upon, and the quantitative determination of β -carotene is quite feasible. The potential potency in I.U. per gm. can therefore be readily calculated. Whether or no this potency is realisable in animal experiments is another matter.

The 1934 Conference (11), recognising the usefulness of the spectrophotometric method for determining vitamin A, recommended "that a sample of cod liver oil, the potency of which has been accurately determined in terms of the International Standard Preparation of β -carotene, shall be provided as a Subsidiary Standard of Reference," and the *United States Pharmacopoeia* Reference Cod Liver Oil (accurately assayed in terms of the 1931 Standard carotene) was deemed suitable. The spectrophotometric method of determining vitamin A (absorption maximum near 328 m μ) was regarded as trustworthy. The intensity of absorption $E_{1\text{cm.}}^{1\%}$ could be measured to within ± 2.5 per cent, and as a means of converting $E_{1\text{cm.}}^{1\%}$ values into a figure representing International Units per gram, the factor 1600 was recommended. The conversion factor was based on comparable and independent tests on the unsaponifiable fractions of liver oils and concentrates of high potency. After seven years it is very difficult to be sure that this factor can be improved upon.

Co-operative tests carried out under the auspices of the Medical Research Council and Lister Institute (Vitamin A Sub-Committee) on samples of (a) halibut liver oil and (b) vitamin A β -naphthoate, tested against the standard β -carotene, have been subjected to statistical examination; conversion factors of 1570 and 1600 to 1700 have been obtained for the oil and the crystalline ester respectively (6, 7, 12). The same group of British workers also tested the U.S.P. Reference Cod Liver Oil and obtained a conversion factor of 1820.

In the United States of America a conversion factor of 2150 is much favoured, and it is desirable to ascertain how the discrepancy has arisen. In England the β -carotene Standard Preparation has been widely used, whereas in America the U.S.P. Reference Oil has been used in the majority of bioassays. The oil was assigned a potency of 3000 I.U. per gm. when first issued (based on a good group of assays collected by Dr. Nelson, ranging from 2400 to 3725). The later assays, carried out in England using the β -carotene standard, indi-

cated a rather lower potency (6, 7): range, 1334 to 3270 I.U. per gm. (10 laboratories), 2200 to 2600 I.U. per gm. (6 out of 10 laboratories), weighted mean 2619 I.U. per gm.; mean limits of error per experiment ($P = 0.99$), 73 to 137 per cent; contemporary $E_{1\text{ cm.}}^{1\%}$ 328 $m\mu = 1.40$ to 1.44 (on nonsaponifiable fraction); conversion factor, 1820. The oil has however been used continuously at a nominal potency of 3000 I.U. per gm., and conclusive evidence of deterioration is difficult to obtain from bioassays. Spectroscopic evidence however seems to be less equivocal, as is shown in Table I.

TABLE I

REPRESENTATIVE DATA ON THE U.S.P. REFERENCE COD LIVER OIL I

$E_{1\text{ cm.}}^{1\%}$ 617 $m\mu$ (Antimony trichloride colour test)	$E_{1\text{ cm.}}^{1\%}$ 325 $m\mu$ (Ultraviolet absorption)		Date	Reference
	On oil	On nonsaponi- fiable fraction		
4.85	1.74	1.58	1934	(13, 21)
4.40	1.55	1.39	1937	(13, 14)
	1.61		1936	(9)
	1.545		1937	(8)
	1.59		1940	(15)
	1.62	1.346		
	1.555	1.38-1.39	1940	(16)
	1.495	1.49*	1941	(17)

* Probably in error.

The clear physical evidence of deterioration indicates that if 3000 I.U. per gm. was the original potency, 2600 I.U. per gm. is a better figure for the last three or four years.

In comparing bioassays and spectrophotometric readings when the U.S.P. oil has been used as a reference substance, it is essential that the physical tests be carried out on the nonsaponifiable fraction of the reference oil (or products of similar potency) lest irrelevant absorption falsify the comparison. For high potency oils, irrelevant absorption is usually a negligible fraction of the gross absorption at 325 $m\mu$ (see Table II).

Table II is based on a large-scale collaborative study and the scatter of the spectrophotometric data is due partly to varying quality of equipment, but the results show beyond any doubt the need for carrying out such tests on the nonsaponifiable fraction of the reference oil,

as the entire range of observations on the oil itself is too high for a linear relationship between potency and absorption.

TABLE II*

	Relative potencies from bioassays	Expected $E_{1\text{ cm. } 328\text{ m}\mu}^{1\%}$	Observed $E_{1\text{ cm. } 328\text{ m}\mu}^{1\%}$	
			Mean	Range
U.S.P. oil.....	1	1.39	1.545	1.43- 1.66 (on oil)
Halibut liver oil.....	23.3	32.4	30.31	28.35- 33.3
Distillation product..	100	139	141.98	134.1 -152.8

* Data based on reference (8).

A later paper (15), with internal evidence of good spectrophotometric technique, affords the material for Table III.

TABLE III

Oil	$E_{1\text{ cm. } 325\text{ m}\mu}^{1\%}$	Relative intensities of absorption	Relative potencies by bioassays	Difference, per cent
U.S.P. Oil I.....	1.35	1	1	
	(on nonsap.)			
Halibut liver oil.....	31.46	23.3	21.66	-7
Mixed liver oil.....	78.68	58.28	61.76	+5.6
Mixed liver oil.....	128.60	95.28	98.36	+3.1
Halibut liver oil.....	15.05	11.14	10.63	-4.6
Mixed liver oil.....	39.85	29.52	29.26	-0.9

This work goes far to establish the validity of a single conversion factor for a wide range of natural liver oils. A factor of 2158 can be obtained by dividing the nominal potency of 3000 I.U. per gm. by 1.39. If, however, the true potency is 2500 I.U. per gm., the conversion factor will be 1800.

Pure vitamin A (18, 19, 20) probably possesses the following characteristics:

$E_{1\text{ cm. } 617\text{ m}\mu}^{1\%} = 6000 \pm 200$ (antimony trichloride colour test)
 $E_{1\text{ cm. } 325\text{ m}\mu}^{1\%} = 1880 \pm 40$ (ultraviolet absorption);
 potency, 3.0 to 3.3×10^6 I.U. per gm. (rat test against β -carotene); conversion factor, 1600 to 1750.

The original conversion factor of 1600 does not, therefore, seem to have been much in error, judging from subsequent British tests against β -carotene. American publications, without adding to the evidence

for the absolute value of the factor, have greatly increased the evidence in favour of an accurately linear relationship between values of the absorption coefficient, $E_{1\text{cm}}^{1\%}$, and potency.

It is obvious, however, that the presence of biologically inert material which absorbs appreciably at 325 m μ must be avoided when this is possible. Unfortunately some commercial products such as whale liver oil (21, 22) and some crude fish oils contain substances of this kind which are not eliminated by saponification. The non-saponifiable fraction may contain vitamin A₂, oxidised vitamin A (23), cyclised vitamin A, or hypothetical vitamin A isomers. In such cases the conversion factor obtained by dividing $E_{1\text{cm}}^{1\%}$ values into biologically determined potency may vary from 800 to 1200 (24).

In addition to extraneous absorption, a further contribution towards a lowered conversion factor would occur if vitamin A alcohol were less potent than the natural esters. A loss of potency in saponification has been recorded several times (25, 26, 27, 28) and German workers (25) have recorded conversion factors of about 3600 for an ester concentrate and 1800 for an alcohol concentrate. In this investigation (25) the method of assay was hardly suitable for absolute determinations, but the 2:1 ratio is less questionable. Whatever the reason for these results, it now seems clear (20)

that there is no difference in the effect on the growth of vitamin-A-deficient rats of equal quantities of vitamin A alcohol or natural vitamin A esters, when these quantities are estimated by measurement of the spectrographic absorption at 328 m μ this result was probably due to the freedom of the preparations tested from oxidation products and other impurities which (may) interfere with the spectrographic estimation.

Experiments on pigs also showed that free and esterified vitamin A are "equally or nearly equally effective."

For the avitaminotic rat, 0.6 μ g. of pure β -carotene and 0.3 μ g. of pure vitamin A alcohol (or an equimolecular amount of ester) are equal to 1 I.U. Assimilation of either preformed vitamin A or β -carotene is normal in the presence of fat (glycerides, sterols, etc.), but it appears that conditions are specially favourable in fresh unhydrolysed fish liver oils, and unfavourable in crude concentrates. The analytical problem therefore resolves itself into two separate aspects: (a) determination of vitamin A content, irrespective of whether the diluent contains materials favouring or hindering assimilation, and (b) determination of the nature, proportions, and properties of "impurities" capable of influencing assimilation. The knowledge needed for the

above is incomplete. Vitamin A preparations are likely to be normal, however, if the following conditions obtain: (a) if in the ultraviolet absorption spectrum there is no fine structure (i.e., cyclised vitamin A absent) and no superimposed general absorption (i.e., absorption band symmetrical and persistent), and (b) if in the antimony trichloride colour test the maximum at 617 m μ is well-defined, the value of $E_{1\text{ cm.}}^{1\%}$ being nearly twice that at 580 m μ (28). Many of the difficulties arise from drastic processing and are likely to diminish in importance as the methods of working up fish livers improve (29, 30).

It might be argued that, although high accuracy in determining vitamin A is desirable because of the money values involved in a great industry affecting public health authorities, it savours of hair-splitting in a more strictly scientific sense. Matters of deeper import are concerned, however. The analyst determines the proportion of a substance present, whilst the bioassayist determines an effect. The distinction, though elementary, is fundamental. Thus while avitaminotic rats use β -carotene as efficiently as vitamin A, the efficiency of conversion of β -carotene in the pig is at best no greater than 30 to 40 per cent. Cattle, sheep, and horses are also less efficient in this respect than rats (31, 32) and the four species appear to require daily either 5.3 to 7.1 $\mu\text{g.}$ of vitamin A per kg. body weight or 24 to 34 $\mu\text{g.}$ of β -carotene per kg. body weight. The ratio of the respective efficacies of vitamin A and carotene widens as the dose is increased and this is probably true also for man (33, 34, 35, 36). The suggestion (32) that separate standards for vitamin A and provitamin A may be needed sooner or later cannot lightly be dismissed. The suggestion is made that following absorption, β -carotene and vitamin A are about equally effective, but roughly 50 to 70 per cent of the former is excreted unchanged in the faeces (36a).

Bioassay of vitamin A.—Opinions differ concerning the precision attainable either in comparing a vitamin A preparation with β -carotene or with a standard liver oil. Collaborative tests on the U.S.P. reference oil tested against β -carotene indicated mean limits of error per experiment ($P = 0.99$) of 73 to 137 per cent (6). A laboratory so organised that all work is secondary to the routine assay of vitamin A (37) reports six years' experience (346 tests involving 3053 pairs) of the diets and technique described by Morgan (38). The limits of error (for a three-weeks test period involving the use of twenty rats and for $P = 0.99$) were 74 to 135 per cent [but see (6)]. This is contrasted with 30 to 339 per cent quoted from a "Report on

the Accuracy of Biological Assays" issued in 1936 (39) for tests involving a vitamin A-free diet (40) recommended by the *British Pharmacopoeia*. Replacement of dextrinised rice starch in this diet by 30 per cent of coconut-cake meal makes it equal in all respects to the vitamin A-free diet referred to above, and it has been suggested that the coconut meal contains some substance intimately concerned with the absorption and utilisation of vitamin A and β -carotene (37). It is further claimed that lack of this hypothetical material causes much slower growth during the depletion period and smaller growth responses to small doses of vitamin A during the test period, and β -carotene is said to be used less efficiently than vitamin A. The view is advanced that vitamin A requires for its action some other material, "probably protein by nature," the formation or supply of which is limited when caseinogen or meat meal forms the sole source of protein in the diet. Palm-kernel meal and dried acetone-extracted herring roe can replace coconut meal, but ground nut-cake meal is ineffective. The herring roe preparation is at least three times as active as coconut meal. Experiments in which 40 International Units were supplied daily to rats showed that the composition of the basal vitamin A-free diet can exert a marked influence on the utilisation of vitamin A for growth purposes and also on the absorption of β -carotene and on its subsequent conversion into vitamin A. A number of attempts have been made to confirm these important claims (unpublished data), but little success has so far been obtained. Further light on this controversial issue will be awaited with interest.

From another point of view, the complexity of the vitamin A problem has been sharply demonstrated (41). In rats restricted to a diet deficient in both vitamins A and E, the reserves of vitamin A were used up much more rapidly than in control animals given synthetic racemic α -tocopheryl acetate. Prolonged deficiency of vitamin E led to secondary deficiency of vitamin A as indicated by its disappearance from the liver. The deficient rats also developed a blanching of the teeth widely regarded as due to a deficiency of vitamin A, but which may perhaps be considered to be a common result of deficiency of either of these vitamins. It is possible that, under physiological conditions, the tocopherol may protect vitamin A against oxidative wastage.

After consumption of alcohol there is always some increase in the vitamin A content of blood, which may often be doubled during an evening (42). Apart from this mobilisation of liver reserves, a diurnal

rhythm in the vitamin A content of the blood has been noted (43). Rats possessing moderate reserves of vitamin A have been placed on a low-vitamin diet and injected with colloidal solutions of polycyclic hydrocarbons (44). The rate of disappearance of vitamin A from the liver is markedly increased by benzpyrene, benzanthracene, methylcholanthrene, and particularly dibenzanthracene. With injection of the latter, the disappearance of vitamin A from the liver was accompanied by a tenfold increase in the vitamin A content of the non-hepatic tissues. The action of the hydrocarbon in releasing vitamin A is similar to that of alcohol in the human, but is much more drastic.

Investigation of the validity of visual dysadaptation as an indicator of the state of vitamin A nutrition continues to give conflicting results. In one study (45) the presence of a dietary avitaminosis became evident after the first day of absence of vitamin A from the diet. In another (46) the first departure from normal dark adaptation was observed on the fourth day of the deficient diet, that is, at a time when no deficiency would have been foreseen according to older ideas. These results imply difficulty in mobilising the stored vitamin of the liver. Diametrically opposed results were reported by two workers experimenting upon themselves (47), since after five weeks on a diet deficient in vitamin A neither showed measurable night blindness. It seems certain (48) that some persons, irrespective of the magnitude of their liver reserve, respond promptly to a deficient diet, whilst other persons continue to exhibit normal dark adaptation for periods up to twelve weeks. Those who readily become dysadapted recover only slowly on a normal diet heavily supplemented with vitamin A (49, 50). The transport of vitamin A may possibly suffer blockage either at the stage of transference from the liver to the blood stream or from the blood stream to the retina. It was found, in seventy-one subjects, that biophotometer readings and fasting blood plasma levels of vitamin A showed only slight correlation (48); nine subjects were retested after supplementing their diet with 25,000 I.U. of vitamin A for five days. The analytical results [based on improved methods (51)] for plasma were 138 to 62 μ g. of carotene, 263 to 96 U.S.P. units of vitamin A per 100 ml., or 493 to 200 U.S.P. units of vitamin A activity. In this field again there is conflict of experience, good correlation between response to a new visual test (52) and the vitamin A content of blood being obtained (53). Subjects suffering from xerophthalmia generally (54) but not always (55) have little or no vitamin A in the blood; similarly persons chosen from low-income groups (and pre-

sumably receiving suboptimal amounts of vitamin A in their food) seem to show low blood vitamin A (56, 57, 58). On the other hand, patients with clinical symptoms of vitamin A deficiency may exhibit a higher content of vitamin A in the blood than apparently healthy subjects (59). The assumption that the magnitude of the liver reserve does not determine the blood vitamin A level has been advanced to account for the poor correlation. Even with persons receiving known amounts of vitamin A for several months, the value of blood vitamin A determinations in assessing nutritional status is uncertain, although a general parallelism between the intake of vitamin A and the blood value is discernible (60). There is probably a critical value (possibly for both carotene and vitamin A) corresponding with minimal requirements, but this appears to vary with different species. Thus (61) for cattle plasma, 25 $\mu\text{g.}$ and 16 $\mu\text{g.}$ per 100 ml. represent estimates for carotene and vitamin A, respectively. These levels are relatively high and should present no difficulty to the analyst, so that recognition of deficiency is feasible; for other species the critical level may be so low as to render the determination of blood vitamin A uninformative. A careful and prolonged test on dogs (62) showed that, during a depletion period of over a year, blood vitamin A ceased to be detectable (<5 I.U. per 100 ml. of blood) in about 150 days, but the animals maintained their weights and remained healthy for another ten months before being killed. The liver reserves were then found to be near exhaustion. Before the depletion period, the dogs received vitamin A for several weeks at dose levels of 100, 400, and 2000 I.U. per kg. per day, and the values for vitamin A in the blood were found to run roughly parallel with vitamin A intake. It is concluded that there is no danger of avitaminosis-A in dogs so long as even traces of vitamin are detectable in the blood of the fasting animal (cf. 92, 96).

The problem of vitamin A in relation to blood is clearly a complex one and the analytical procedure needs to be scrutinised carefully. A well-calibrated technique has been described (63) which makes use of photoelectric colorimetry applied to solutions in petroleum ether. The solutions are obtained by simple extraction of serum or plasma precipitated with alcohol. The results obtained with twenty-three subjects (free from visual dysadaptation) varied from 50 to 241 $\mu\text{g.}$ of carotene and 72 to 157 I.U. of vitamin A per 100 ml. of serum or plasma, confirming earlier data (64). A possible seasonal effect is envisaged since the February-March data were in general lower than those obtained in the autumn.

Defective growth arising from deficiencies other than vitamin A in vitamin A-free diets has again been noted (65). The absorption and retention of carotene and vitamin A by hens varies with fat intake (66). The presence of 4 per cent of fat in the diet has a more marked effect on the absorption of carotene than of vitamin A. After five weeks treatment with massive doses of vitamin A, the livers of birds on a low fat ration (<0.1 per cent) contained only one-eighth as much vitamin as those from birds given a normal fat ration. A substance extractable from the normal ration by means of ether is therefore held to be necessary for the retention of vitamin A. To what extent such a concept overlaps with the idea of antioxidants is uncertain but a distinction has been drawn between antioxidants which function *in vitro* and those which are effective in the intestinal tract (66a). Thus carotene in linoleic ester is stabilised by soybean oil and especially by the first 1 per cent of material obtained in the molecular distillation. The effectiveness of soybean oil is not lost by oxidation to peroxide number 40, whilst γ -tocopherol or catechol, but not hydroquinone and pyrogallol, can replace the oil.

Secondary or "conditioned" deficiency of vitamin A is shown by lowered liver reserves in almost all diseases (67). In accidental death the median reserve was found to be 220 I.U. per gm., in hernia 160, in pneumonia 63, and in chronic nephritis 25. Visual dysadaptation responds badly to vitamin A therapy in urolithiasis (68), cirrhosis of the liver (69), thyroid diseases (70), and diabetes (71). The latter disease is associated with carotenemia and increased liver reserves of vitamin A, so that faulty dark adaptation must in some pathological conditions be due to abnormal metabolism of both preformed and provitamin A rather than to dietary deficiency or depleted reserves (56).

A careful psychophysiological study of "night blindness" in fifty-two soldiers (72) has led to the conclusion that the condition as seen in England, though phenomenologically the same, is aetiologically quite distinct from night blindness due to nutritional causes. Most of the cases studied were probably of psychological origin. The value of dark-adaptation tests for the discovery of night blindness and as an indicator of vitamin A deficiency is regarded as doubtful, and it is said to be probable, though hitherto unproven, that the results of dark-adaptation tests may be influenced by mental mechanisms.

Vitamin A requirement.—In spite of the great expansion in recent years of the fish liver oil industry (30) the majority of human beings must continue to depend, as heretofore, more on carotenoid provita-

mins A than on preformed vitamin A. Experiments on the absorption of carotene are therefore of immediate practical importance as well as scientific interest. A valuable monograph (73) contains new observations and a careful survey of the literature. The word "absorption" covers all losses occurring during the passage of carotene through the alimentary tract and is measured by subtracting from the amount of carotene administered the amount recovered from the faeces. One worker (74) records 30 to 70 per cent (round figure 50 per cent) "absorption" by humans. A marked increase in serum carotenoids follows ingestion, for a few days, of carotene in oil or carrot puree. The latter is a little better utilised than the former. Experiments on rats (74, 75, 76, 77) also indicate 50 per cent as a rough estimate of "absorption."

A well-planned study (33) led to the conclusion that 2500 I.U. per day of vitamin A or 5000 I.U. of β -carotene are sufficient to meet the normal human daily requirement. Ten adult males were maintained for 188 days on a diet low in vitamin A activity, and all showed loss of weight, visual dysadaptation, and changes in the blood cells. Half of the subjects were then given β -carotene in oil and half the proprietary vitamin A ester concentrate, "Vogan." Apparently the potency of "Vogan" was calculated using an excessively high conversion factor (25) and it is very probable that most workers would regard the material as overvalued by a factor of 2. On this basis the daily requirement of preformed vitamin A will be nearer 1250 I.U., and 0.375 mg. of vitamin A ($1250 \times 0.3 \mu\text{g.}$) will be equivalent to 3.0 mg. ($5000 \times 0.6 \mu\text{g.}$) of β -carotene. Earlier work (34, 35) showed that normal dark adaptation requires 25 to 55 I.U. per kg. of bodyweight of vitamin A and 43 to 103 I.U. per kg. of β -carotene (i.e., 1750 to 3850 I.U. preformed vitamin A and some 3000 to 7200 I.U. of carotene for a 70 kg. man) but appreciably lower estimates have been made (73).

Pigs fail to make use of zeaxanthin (20) but need 66 I.U. and 22 I.U. per day per kg. bodyweight, of β -carotene and vitamin A respectively. Large doses of vitamin A over a long period induced no toxic symptoms in cows and brought about a 10 per cent increase in milk production accompanied by a slight rise in fat content and some transfer of additional vitamin A to the milk (78). The need for supplementary vitamin A is probably much greater during lactation than during pregnancy, since the daily output in milk may reach 3000 I.U. per day (79). Recent work on carotene and vitamin A in butter (80,

81) provides confirmation of this, and the work is of particular interest from the analytical standpoint.

Vitamin A in urine.—Since vitamin A is fat-soluble rather than water-soluble its absence from normal urine (82, 83, 84) is to be expected. It has however been detected in human urines from patients suffering from cancer (85), chronic nephritis, nephrosis, pneumonia, cirrhosis of the liver, and icterus with closure of the bile duct (86, 91), tuberculosis, and chronic infections (87). Excretion is conditional neither upon renal damage nor dietary excess of vitamin, but the suggestion is made that shortage of ascorbic acid is a relevant factor. In pneumonia, excretion may be as high as 3000 I.U. daily until the crisis has been passed when it ceases abruptly (86, 88) and the low value for blood vitamin A, which accompanies urinary excretion, returns to normal. Chronic nephritis is generally accompanied by high blood vitamin A and considerable urinary excretion (90) but for most of the diseases mentioned vitamin A need not necessarily appear in the urine. If it does, the blood vitamin A is usually, but not always, subnormal (90). Urinary excretion of vitamin A occurs in only a small proportion of patients suffering from skin diseases. Vitamin A is apparently present in some normal pregnancy urines (96) but less frequently in England than in Germany (95). Evidence has been advanced to show that urinary excretion of vitamin A is connected with injury to or blockage of the reticulo-endothelial system (97, 98, 99).

Vitamin A was not found in the urine of rats, whether healthy or diseased (96), nor in that of rabbits (92, 93), except to a minute extent in an animal infected with sputum from a human patient, affected with pneumonia (76). Attempted blockage of the reticulo-endothelial system by injection of bismuth did not result in urinary excretion of vitamin A by rats and rabbits. The remarkable observation that the healthy dog excretes quite large quantities (90 to 450 I.U. per 100 ml.) of vitamin A in its urine (92, 93, 96) is of great interest and is determined, so far as can be judged, by factors other than the day-to-day supply in the diet. An important investigation (96) shows that the material excreted satisfies all the accepted criteria for vitamin A since it shows the ultraviolet maximum at 325 m μ and the 620 m μ maximum in the colour test, and restores growth in avitaminotic rats.

Protein, not necessarily heat-coagulable, is always present in urines containing vitamin A. Such urines, unlike normal human urines, are capable of dissolving more vitamin A on shaking with halibut liver oil. When protein is being excreted, but not vitamin A, the urine acts to

an appreciable extent as a solvent for vitamin A. The excretion of vitamin A is highly selective, the concentration per unit of lipid being in the urine at least one hundred times greater than in blood, and it is significant that vitamin D is virtually absent from human urine and from that of a dog given calciferol. Kidneys taken at autopsy from victims of accidents normally contain about 190 I.U. per 100 gm. of vitamin A, whereas the values obtained from cases of death from pneumonia or chronic nephritis are about 40 to 50 I.U. per 100 gm. Liver reserves are reduced in such cases, thus in pneumonia the median normal liver store of 330,000 I.U. falls to a median value of 94,000 I.U.; even with urinary excretion at the high rate of 3000 I.U. per day, much of the vitamin which disappears cannot be traced.

VITAMIN D

Alternatives to the bioassay.—The vitamin D potency of fish liver oils may reach 200,000 I.U. per gm. in exceptional cases, but 100 I.U. per gm. is more typical. Since calciferol has a potency of 40×10^6 I.U. per gm. the real analytical problem involves the determination of a minor constituent with an accuracy of a few per cent even though its concentration ranges from 0.0001–0.5 per cent. This is a formidable problem, complicated by the fact that the vitamin D is accompanied by much larger quantities of sterols, and often of vitamin A.

A new method applied to tunny and halibut liver oils seems promising (100). It involves chromatographic adsorption of nonsaponifiable matter from heptane solution on a column of "Hydriffin K₄" (an active carbon from Lurgi G.m.b.H., Frankfurt a.M.). The chromatogram is developed with heptane and vitamin D passes quickly through the column; elution should be stopped as soon as vitamin A appears (detected in the eluate by means of the antimony trichloride colour test), and vitamin D is determined in the eluate by means of the ultraviolet maximum at 265 m μ . The report is not unconvincing, but the writer and other workers have failed to use the method successfully. The War unfortunately makes it impossible to communicate with the author or to obtain the adsorbent used by him. It is however very unlikely that the method can serve for oils less potent than 4000 I.U. per gm.

The fact that a solution of antimony trichloride in chloroform gives rise to a good colour test with calciferol is well known. A new reagent containing a small quantity of acetyl chloride (101) possesses many advantages. Cholesterol and provitamins D also give rise to

colour with antimony trichloride but by reason of the fact that the colour with calciferol is considerably intensified by the new as compared with the old reagent, interference is not quite so important. Unfortunately the time taken for the colour reactions to develop is an important factor. This work is promising but still full of difficulty. Another investigation (102) introduces a significant preliminary treatment of the nonsaponifiable fraction with maleic anhydride to destroy vitamin A, carotenoids (if present) and some sterols. This undoubtedly tends to limit interfering processes in the colour test. Within rigidly defined limits progress of an empirical kind is being made, but there is still much to be done before any alternative to the biological method of assay can command general acceptance.

Results from nineteen laboratories for the assay of oil containing vitamin D by the preventive biological method have been recorded (103) and examined statistically (104). In the chick method of assay a group ashing procedure is as trustworthy as the more tedious individual ashing and simple proportionality holds between percentage of bone ash and body weight for vitamin D supplements over the range 0 to 30 I.U. per 100 gm. of feed (105).

Numerous workers have reported on parenteral and oral vitamin D shock therapy, and prophylaxis (113, 114). Single doses of 7.5 to 25 mg. of vitamins D_2 and D_3 have been used successfully for summer rickets (106), winter rickets, and spasmophilia (107), florid rickets (108, 109, 110), and tetany (111). Metabolic effects were mostly satisfactory (111) and neither toxic effects nor hypercalcaemia were observed (112). Vitamin D_3 was a little more effective than vitamin D_2 (107, 110). No pathological changes were observed at autopsy in ten children who died (from pneumonia or toxæmia) from 2 to 58 days after massive doses of vitamin D_2 or D_3 (115). Parenteral shock prophylaxis (15 mg. D_2 or D_3) gave good results in forty-six premature infants. Oral treatment was also effective (116).

Rats respond very much better to vitamin D_2 administered orally than parenterally (117). Vitamins D_2 and D_3 (10,000 I.U. per day) are highly toxic to dogs, but excess of vitamin A affords measurable protection against hypervitaminosis D (118).

VITAMIN E

Standardisation.—An important co-operative test of synthetic racemic tocopheryl acetate as a possible international standard for vitamin E has been completed (119a) under the auspices of a British

Committee. Workers in seventeen laboratories were asked to test four solutions of graded strengths (disclosed but not identified). The object was to obtain the relation between dosage and response, the response used being the fertility-rate defined as the percentage of positively mated female rats which produced a litter. Vitamin E deficiency is a condition which in an individual animal is not cured in a smoothly graduated series of stages; for statistical purposes the response is treated as of an all or none, not of a graded, type. However, the response *vs.* dosage can, as is usual in such cases, be transferred into a linear relation by plotting the normal equivalent deviation (or probit) of the percentage response against the logarithm of the dose.

Arrangements were made whereby the stability of the feeding solutions after the tests and of the original material after keeping was tested spectrophotometrically. All the solutions kept well. Thirteen of the seventeen laboratories invited, completed the biological tests and sent in detailed reports for statistical analysis. In four of the laboratories the slope of the dosage-response curve proved not to differ significantly from zero; in other words the responses to the graded doses were not themselves significantly graded. The data from the remaining nine laboratories led to the following conclusions. The median fertility dose (that dose which enables 50 per cent of the rats used to bear a litter) varied from 0.5 mg. synthetic racemic tocopheryl acetate to 1.71 mg. (average 0.986). The great variation in the size of the median fertility dose brings further evidence, if that were needed, of the necessity for establishing an international standard for vitamin E.

The accuracy of the biological technique, as shown by the limits of error (689 rats, $P = 0.99$) of 72 to 139 per cent affords a satisfactory basis for recommending the synthetic compound for adoption as an international standard.

A synthesis of *dl*-5,7-diethyltolcol has been announced (119). After purification through the allophanate it is obtained as a viscous strongly reducing oil. It is only a little less active than 5,7-dimethyltolcol, and about as active as β -tocopherol (5,8-dimethyltolcol). α -Tocopherylquinone, 2,3,5-trimethyl-6-phytyl-1,4-benzoquinone and 2,3,5-trimethyl-6-(dihydrophytyl)-1,4-benzoquinone show no vitamin E activity, but naphthotocopherol (prepared from vitamin K) is about a third as potent as β - or γ -tocopherol (120).

An improved method has been reported (121) for determining the tocopherol content of oils by a modification of the iron-dipyridyl

method of Emmerie (122). Interfering substances are eliminated by treatment with 85 per cent sulphuric acid, followed by 1 per cent potassium hydroxide.

The Furter & Meyer colour test, involving oxidation with nitric acid, has been studied spectrophotometrically (123). An absorption maximum at 470 m μ is shown by different tocopherols, but the intensities of absorption (molecular extinction coefficients) diverge considerably. Oxidation of α -tocopherol with silver nitrate or ferric chloride results in inactive, spectroscopically identical products; the *o*-quinone obtained by the action of nitric acid shows an absorption maximum at 405 m μ and is less active than the parent substance (124).

The successful use of vitamin E in the treatment of thirty cases of primary fibrositis (125) is interesting, especially since two cases of slight response to wheat germ oil (3 cc. daily for four weeks) obtained marked relief in one week with large doses of a fraction, rich in α -tocopherol, obtained by the process of "molecular distillation." Atrophic arthritis with secondary fibrositis differed from hypertrophic arthritis with secondary fibrositis, in that a decrease in muscle soreness and stiffness followed vitamin E therapy exclusively with the former, and then only in eight cases out of twenty.

α -Tocopherol has quite definite growth stimulating activity (126) even with prematurely born infants (127) and possesses prophylactic value in preventing nutritional myopathy (128). Four generations of experimental animals reared on a diet low in vitamin E, exhibited deficiency symptoms at an earlier stage in each succeeding generation, and maturity in the females was progressively delayed; all males were sterile, but in even the fourth generation they became fertile after administration of germ oil (129). Avitaminosis E in female rats is described (130) as causing in the uterus rapid and persistent hypertrophy, muscular degeneration and yellowish pigmentation. A vitamin E-deficient diet on which rats become sterile permits reproduction in goats, but the vitamin cannot be detected in the goat milk-fat, adipose tissue, or muscle (131).

Young chicks develop nutritional encephalomalacia on a natural ration treated with ferric chloride in ether to destroy vitamin E if heat is used to evaporate the ether, but not if the ether is allowed to evaporate spontaneously in the cold. The destruction of vitamin E is a necessary preliminary so that the disease requires the lack of some heat labile material or some failure to utilize other substances when

vitamin E is absent (133). Prolonged feeding of newly hatched chicks on the ration treated as above brought about a condition resembling anaemia. Destruction of red blood cells accompanied by deposition of an iron compound, probably hemosiderin, occurred in the liver. Whilst the cause of the erythrophagocytosis is not clear, it is suggested that treatment of the food destroys not only vitamin E but also some other substance essential to the red blood cells. This material is available in cod or sardine liver oils, but not in halibut liver oil (132, 134). Castrated male fowl reared on a diet deficient in vitamin E respond to injections of testosterone propionate much more rapidly and effectively when the diet is supplemented with α -tocopherol. In the fowl some vitamin E in the diet is necessary to ensure the most effective utilisation of the male hormone (135).

Chicks exhibiting nutritional encephalomalacia sometimes show pronounced oedema. This is often associated with abnormal cholesterol metabolism; therefore the brains of normal and affected chicks might be expected to differ in cholesterol content. The sterol can be extracted with chloroform and determined with some accuracy by photoelectric methods applied to the Liebermann-Burchard colour reaction. A decrease in the cholesterol content of diseased as compared with normal brains during the third week and thereafter rapidly becomes more marked.

In the normal brains the cholesterol content rises after the second week but fails to do so in the brains of chicks on a diet deficient in vitamin E. A cholesterol disturbance also occurs in dystrophic rabbits and in women subject to recurrent abortion. Vitamin E must therefore be closely connected with normal cholesterol metabolism (136).

The reported effects of vitamin E deficiency in experimental animals have been summarised (137). The different effects of cod liver oil and halibut liver oil referred to above are tentatively ascribed to variability in the effects of different forms of vitamin D. A survey of the extensive list of manifestations of vitamin E deficiency shows that this vitamin "is necessary for normal and efficient utilization of chemical substances of the anthracene group"—sterols, vitamins D, androgens, oestrogens and progesterone (cyclopentanophenanthrene group?). This view enhances the importance to be attached to vitamin E, and a "stimulus to further investigation appears in the fact that the tocopherols are closely related to antioxidants."

Nutritional muscular dystrophy in rabbits and rats is prevented or cured by α -tocopherol (138) without addition of a water-soluble

factor. The antidystrophy factor does not appear to be multiple in nature. The curative dose for rabbits is 0.2–0.4 mg. per kg. daily (140).

Recent clinical experience (141 to 145) of vitamin E therapy in neuromuscular disease reveals no significant difference from the course of the untreated disease. A leading article (146) contains the challenging statement

.... the place of vitamin E in the index of treatment will not be finally decided until simple means are available for analysing the amount of the vitamin in the body fluids and tissues. We do not doubt that vitamin E is an important food factor, but we find nothing yet to persuade us that any human disease or any disturbance of childbearing or locomotion in man is due to deficiency of vitamin E or is alleviated by treatment with it.

Just in its demand for further evidence though the above blunt verdict may be, the cure of nutritional muscular dystrophy in animals by α -tocopherol is abundantly proved.

VITAMIN K

In the short period 1935 to 1940 the existence was established of a vitamin required by the chick for maintenance of normal blood clotting power, and methods of bioassay worked out, the distribution of the vitamin was ascertained and the best sources recognised; the two compounds, vitamins K_1 and K_2 , were isolated and their structures determined. Synthesis soon followed and in the course of the work simple and readily accessible substances were found to possess high vitamin K activity. The term vitamin K now covers an extensive group of compounds, and the list is probably still incomplete. The physiology of vitamin K has been studied with success and considerable experience of vitamin K therapy gained. This astonishing achievement is authoritatively reviewed (147) in papers with a bibliography of nearly two hundred references. The rapidity with which the whole problem has been to a great extent solved is a significant demonstration of the mobilisation of (largely American) scientific resources in a beneficent blitzkrieg.

Bioassay.—Although the necessity for standardisation assays is less acute for vitamin K than other fat soluble vitamins, an official quantitative procedure is desirable. Progress in this direction has been made (148). The basal diet (cf. also 147), management during the period of preparation, and method of administering supplements are recorded. Blood-clotting time is abandoned in favour of “pro-

thrombin time" which measures blood prothrombin level and in turn the effect of vitamin K supplements on the prothrombin level. A plot of the reciprocal mean prothrombin time against the logarithm of the vitamin K dosage gives practically a straight line. The potency of an assayed supplement can then be calculated by comparison with a reference standard which should not be omitted from the test, as so-called "master" or "response curves" are apt to mislead.

Based on a scale such that 1 unit is defined as the antihaemorrhagic activity of 1 μ g. of 2-methyl-1,4-naphthoquinone, the activities of the following compounds were observed: 2-methyl-1,4-naphthohydroquinone diacetate gave in three tests 470, 507, and 518 units per mg.; 2-methyl-1,4-naphthohydroquinone diphosphoric acid ester tetrasodium salt hexa-hydrate 440, 497, and 507 units per mg.; and vitamin K₁, 303, 308, 294, and 280 units per mg.

Chemical and physical properties.—A colour test for 2-methyl-1,4-naphthoquinone and related products (150) involves the use of 2,4-dinitrophenylhydrazine in hydrochloric acid. The solution is warmed, then cooled and ammonia added, followed by addition of amyl alcohol. A stable green colour appears on dilution with water, which passes into the amyl alcohol phase. Sodium methoxide in methanol can replace ammonia, producing a blue-green colour; amyl alcohol extracts the coloured product, but its use is not essential. The intensity of colour varies directly as the concentration of quinone.

A quantitative reduction-oxidation method for the estimation of vitamin K₁, and similar substances (151), makes use of Raney's nickel catalyst and potassium acetate (phenosafranine indicator) for reduction to hydroquinones. These are then determined by titration using 2,6-dichlorophenolindophenol. The method is useful for determining vitamin K activity in vegetable oils.

In the earlier phases of the work leading to elucidation of the structure of vitamin K, observations on absorption spectra played a useful part (152 to 155). 2-Alkyl and 2,3-dialkyl-naphthoquinones exhibit four well-defined absorption bands near 243, 249, 260, and 269 m μ (maxima) all at approximately the same high intensity as well as a much less intense band near 325 m μ . Recent work on the spectra of quinones (154) indicates that the maxima at 243, 249 and 325 m μ are due to the grouping $-\text{C}_6\text{H}_4\text{CO}-$ and those at 260 and 269 m μ to the quinonoid half of the molecule. Reduction products of vitamins K₁ and K₂ in which the quinonoid part of the molecules remains intact, show only the 260 and 270 m μ maxima. α -Tocopheryl-

quinone (155) contains the same chromophoric grouping and exhibits very similar absorption.

Clinical experience.—The plasma prothrombin level of the newborn infant may fall to dangerously low levels during the first week of life (157, 158). Prophylactic administration of vitamin K to the mother prior to delivery or to the newborn baby is successful, the usual dose in the latter case being 1 mg. This exceeds the minimum daily requirement by one thousandfold (159, 160). Milk contains enough preformed vitamin K to meet this very low requirement. Using 2-methyl-4-amino-1-naphthol ("Synkamin") intramuscularly, maintenance therapy was effective with daily injections of 2 μ g. Prophylactic therapy was successful with single injections of about 20 μ g. within the first few hours of life. With 5 μ g. or more given intramuscularly in one dose maximal curative response occurs in 8 to 10 hours. The slight delay also occurs when the vitamin is given intravenously (158). The fall in prothrombin level which occurs in the first four days of life is soon followed by a rise which develops as soon as breast milk becomes abundant. Dried milk (5 to 10 gm.) in water fed to infants on the third or fourth day gives a good prothrombin response, ether-extracted dried milk is much less effective, and ether soluble material correspondingly active.

If vitamin K (in the form of potassium 2-methyl-1,4-naphthohydroquinone disulphate) is injected into eggs prior to their incubation, the newly hatched chicks store enough vitamin to protect them against the sudden drop in prothrombin level which occurs with a diet deficient in vitamin K (161). General clinical experience (162 to 171) includes the use of vitamin K in haemorrhagic diseases of infants and children and in labour (163). The value of vitamin K preparations in the prevention and treatment of haemorrhage in hepatic and biliary diseases associated with jaundice is now widely accepted.

Defective absorption of vitamin K, due to absence from the intestine of bile salts is the main cause of prothrombin deficiency in obstructive jaundice and biliary fistula prior to operation. Intravenous injection of 2-methyl-1,4-naphthohydroquinone disuccinate works well. A close analogy has been drawn between haemorrhage in jaundice and haemophilia. "In both an upset in the coagulation mechanism exists which is due in the one to prothrombin deficiency and in the other to an unknown congenital cause" (170).

Prothrombin determination does not provide an indication of body reserves of vitamin K. In jaundice or bile fistula, vitamin K-

bile salt therapy is desirable, even if the prothrombin level is normal (171).

Vitamin K-active preparations show pharmacological effects typical of quinones and quinols. For rabbits the lethal dose of 2-methyl-1,4-naphthoquinone is 300 to 500 times the minimum dose needed to obtain vitamin K activity in chicks. The lethal dose for rabbits and mice brings about convulsions and inhibits the oxidation-reduction function of haemoglobin. In the case of 2-methyl-1,4-naphthoquinol disuccinate ("Synkavit") the lethal dose for mice is 275 to 400 mg. per kg. (172).

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NUTRITION

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INTRODUCTION¹

This review deals principally with nutrition in the tropics and the Far East, especially with those aspects of nutrition which are considered very important in these regions; it will be understood, however, that certain special aspects are of equal importance elsewhere, such as calcium metabolism, dental caries, etc. The biochemical phases of these problems are brought prominently forward in this review.

Owing to the limited space available, various subjects have been omitted in their entirety, nor was there space for a critical discussion of many of the papers cited; in such cases the review is confined to a short summary of their contents only.

Although the subjects dealt with are of common interest for many regions in the tropics and in the Far East, it would be impossible to consider these nutrition problems, and especially the applicability of research upon them, from a single viewpoint. The reason for this is that these regions, which include such an extensive area of the earth's surface, differ considerably in climate, density of population, diet, nature of soil, etc. There are, however, certain circumstances which make it possible to speak about nutrition problems which most tropical countries and countries in the Far East have in common, and which—at least in many respects—often differ from those in temperate regions, especially in North America and Europe.

In the fertile regions of the tropics and the Far East, such as

¹ Owing to the war, it was not possible to look up all the literature which might have been important in this review. Following the outbreak of war, many of the European periodicals either did not come to hand at all, or arrived at very irregular intervals. Moreover, many periodicals from other countries only arrived after the lapse of months and appear to have escaped mention in earlier reviews. For these reasons more references are made to literature which appeared in the year 1940 than was first intended. It would be more correct to say that this review refers to the literature received in Batavia during the period January to October 1941. Almost all articles referred to were read in the original form.

China, Japan, India, Java, etc., the population is usually so dense that the individual can only with difficulty find a means of existence. Lack of money and lack of industrialisation, coupled with the fact that the importation of foodstuffs renders many foods very expensive, make the people of these regions more dependent on the products of their own soil than is the case elsewhere. The same applies to the areas which are less fertile, even though less populated.

The combination of these circumstances often makes subsistence economy much more important than monetary economy (which is just the reverse in industrialised regions). As a result, the population becomes principally dependent on locally produced foodstuffs, no matter how unsuitable these may sometimes be from a nutritional point of view.

It very often happens that the best foodstuffs, such as rice and other cereals, are sold in order to obtain money, while less nutritive foodstuffs, such as manioc, are kept for home use. Usually no relation exists between income, diet, and the state of nutrition unless it be in the big towns and industrial areas. Since people are compelled to use cheap foodstuffs, and since other restrictions are imposed by religion, lack of space, lack of cattle food, etc., the menu is often almost exclusively vegetarian.

Relative overpopulation and therefore scanty means of existence, subsistence economy, a vegetarian diet, and malnutrition of children are characteristic of large areas in the Far East and the tropics, even in very fertile regions, a state of affairs contrary to that found in many temperate regions. Furthermore, foodstuffs and vegetables are obviously quite different from region to region, subject to the influence of climate, soil, and custom.

These factors (or at least some of them) and their direct and indirect results indicate the special tasks which await the nutrition worker and the biochemist engaged in nutrition work in these regions. Other factors which should be considered in the nutrition problems of the tropics are the influence of tradition, habits, religion, ignorance, etc., which play a much more important role than in temperate regions.

Diseases such as malaria, intestinal parasitism, etc., considerably reduce the nutritive value of the diet, meagre as it often is.

The nutritionist finds, on the one hand, a rich field for research in the investigation and analysis of foodstuffs not known in temperate regions, in the study of unfamiliar methods to preserve and prepare certain dishes, and in the investigation of deficiency diseases little

known or not met with elsewhere; on the other hand, he will always have to keep in mind the fact that the diet of many inhabitants of the tropics and the Far East is poor, monotonous, and unvaried, especially between crop periods. He will be more interested, therefore, in minimum diets and minimum nutritive requirements than in optimal diets and optimum requirements, whatever may be precisely understood by these terms. Very often the nutrition worker's first concern in these regions will be to provide a quantitatively sufficient diet rather than one which is qualitatively as good as possible. And to a greater extent than his colleagues in the North will he be aware of the relative incompleteness (and sometimes overestimation) of our knowledge of nutrition, and the uncertainty of our standards, as was well pointed out by Cathcart some time ago (1). He will be wondering whether there is not a possibility of the human organism possessing a much greater adaptability with regard to its nutrition than has been assumed thus far.

On account of all these considerations much attention has been paid to nutrition in the tropics in the last few years, to a stock-taking of foodstuffs and nutritional habits,² to influences of climate and race, to testing the so-called nutrition standards and requirements, and to many other relevant problems.³ Moreover, it is felt to be more and more of primary importance that in many regions a system of agriculture more adapted to the dietary needs of the local population should be encouraged rather than one aimed at export and financial gain (2).

FOODSTUFFS

An investigation of the foodstuffs which play a far greater role in the dietary of the tropics and the Far East than in the temperate zones is very important, as well as a compilation of all foodstuffs which are consumed in one particular country or in certain areas.

² Reports of the Committee on Nutrition in the Colonial Empire: 1st Report, parts I and II, London, 1939; also Report of the Intergovernmental Conference of Far-Eastern Countries on Rural Hygiene, Bandoeng (Java), 1937, held under the auspices of the Health Organisation of the League of Nations.

³ Bulletin of the Health Organisation of the League of Nations, 1938, Vol. 8: Report of the Technical Commission on Nutrition, 3rd Session, 1937; also Report by a Special Committee of the Technical Commission on Nutrition, 1938.

Nutrition tables (3) are regularly published in different countries. Fortunately, Latin names as well as local native names are now more frequently used, which makes them comprehensible to the outsider, whereas methods of analysis, description of analysed samples, and methods of interpretation of the tables are being recorded more and more. It is not extraordinary that for one particular foodstuff in different tables often very different analytical values and vitamin percentages are found, since to a greater extent than in Europe and North America consideration has to be given to the many different varieties, the great influence of climate, soil, maturity, etc. It should be noted that methods of preparation, preservation, and cooking, the degree of digestibility and of absorption (bearing in mind also the influence on these factors of the various tropical intestinal infections, malaria, etc.) may invalidate the nutritional values calculated according to the nutrition tables.

In certain diets, especially those containing many different foodstuffs (which is the case in certain tropical regions), it is sometimes possible to classify the foodstuffs into groups, e.g., cereals, fish, leafy vegetables, and to choose group averages instead of separate analysis values—a method which can simplify calculations considerably.

Of all the principal foodstuffs consumed in the Far East, rice is the one that from ancient times has received the most attention, especially in connection with the associated beriberi problem. A number of publications on this subject, all from the Far East (4), and others concerning the combination of rice and fish in the diet (5), appeared recently.⁴ From these and other papers it is evident that one cannot speak about one polished-rice problem only for the whole of the Far East and the tropics. On the contrary, the problem changes with the country, the local habits, and conditions. In India beriberi is found in towns as well as in the country, but there is the favourable factor that a large part of the population in certain areas consumes "par-boiled" rice. In Java the consumption of milled rice and the occurrence of beriberi are, for the greater part, confined to the big towns. The population in the country consumes principally home-pounded rice containing much thiamin, but they dislike the reddish or brownish

⁴ After completion of this review a Bulletin of the Health Organisation of the League of Nations appeared (Vol. IX, No. 3, 1940-41) in which several papers published by W. B. Aykroyd, A. C. van Veen and others on the rice problem are contained.

colour of many of the husked rice varieties. In Japan and some other regions the rice is not only milled, but also polished for preservation and improvement of appearance. In regions where fresh rice is always obtainable, propaganda should be carried on for white husked rice or undermilled rice which has a nicer appearance, while in those regions where the question of preservation plays an important part, the use of parboiled rice should be advocated (to replace milled or polished rice); this is not only of good colour and capable of being preserved (after milling) but also retains a high thiamin percentage after intensive washing. An explanation of this is given by Aykroyd (6). Nicotinic acid is also well retained (7). Aykroyd, as well as van Veen, considers the addition of thiamin to salt, to supplement the deficiencies of polished rice, impracticable in these regions for technical, financial, and other reasons.

It appears from animal tests (8a) that cystine, methionine, and lysine supplement the proteins of husked and polished rice, while tryptophane fails to do so. Cystine, however, does not seem to supplement the proteins of rice bran and rice polishings. Rice bran appears to have no shortage of lysine (9). Polished rice and rice polishings supplement one another in this respect to a certain extent. In another publication (8b) Kik gives data on the cystine, tryptophane, lysine, arginine, and histidine percentages of whole rice, polished rice, rice bran and polishings and comparisons with those of corn, wheat, and casein. The variety of the rice and also the nature of fertilizers used influence the composition of the proteins.

Very interesting is the observation that a certain deficiency disease in foxes, Chastek paralysis, seems to occur as a result of uncooked fish in the ration, but may be prevented by large quantities of thiamin (10). Pathological findings and therapeutic experiments confirm that this paralysis is substantially the result of a thiamin deficiency, though the reasons are not clear nor is the part played by the raw fish in the diet. The writers refer to the occurrence of beriberi in Japan and in the Netherlands East Indies as a result of diets consisting for the greater part of polished rice and fish, and suggest the possibility of a joint cause. Although the occurrence of beriberi is easily explained as a result of the thiamin deficiency of washed polished rice alone, the suggestion seems very interesting.

Manioc tubers (*Manihot utilissima*) are grown and consumed in large quantities in many regions, chiefly because of the great agricultural advantages (abundant production on nonfertile soil). A good

review of the nutritional value of the tubers and leaves of manioc is given by Raymond and others (11). In its raw condition this product, though lacking in protein and fat, and consisting almost exclusively of carbohydrates, also contains appreciable quantities of vitamins (thiamin and ascorbic acid). At the same time, however, it is very often poisonous owing to the presence of relatively stable cyanogenetic glucosides. As to the effect of hydrogen cyanide in foodstuffs on the organism see Clark's publication (12). Dried manioc is usually non-poisonous, but no longer contains any vitamins. Van Veen has indicated that the nitrogen present consists of only about 50 per cent protein nitrogen which means that the protein percentage even in dried manioc tubers is very low, between 0.5 and 0.6 per cent (13).

The taro root (*Colocasia esculenta*) is an important native food in large areas of the Pacific. A good paper on its nutritional value has been published by Potgieter (14).

A publication by Adolph & Liu (15) has appeared on the nutritional value of the sweet potato (*Ipomoea batatas*). The authors found that their human subjects remained in nitrogen balance after a large daily consumption of the tuber.

Lee has made a metabolic study of soybean sprouts, which constitute a favourite ascorbic acid containing food in many countries of the Far East (16). Hsien Wu *et al.* are continuing their series of studies of soybean diets which are so important in a great part of the Far East (17). Fan *et al.* (18) have made a metabolic study of roasted soybean flour, which has been recommended by Guy & Yeh as a very useful food for infants. Fan & Chen have drawn attention to the marked variations in the composition of soybean milk (19). Chi & Adolph have carried out studies to determine the available carbohydrate (20) in leafy vegetables, while Hsu & Adolph have similarly investigated Chinese celery cabbage; these vegetables are of possible value as supplements to a cereal diet (21).

More attention than has thus far been given to the question should certainly be paid to special methods of preparation of some foodstuffs in these regions, either to ensure that they can be preserved for a longer period in the cheapest possible way or to make them more digestible. Examples of this are the preparation in Java, with the aid of fungi, of certain foodstuffs from leguminous seeds and cocoanut presscake.

Nuoc mam is an important liquid fish product in Indo-China, prepared by the autolysis of various kinds of salted fish, partly under

the influence of certain bacteria (22). It has a not unpleasant odour and can be preserved for a long time. Investigations on the mineral constituents of this product were made by Vialard-Goudou *et al.* (23); also on the tryptophane content (24) and that of several other amino acids (25). Van Veen has stated that the volatile products that cause the typical flavour of *nuoc mam* contain methyl ketones (26), which he also found—as well as butylaldehyde—in pedah (a salted, nondried fish product from Siam, Borneo, and the Straits Settlements, made from *Scomber kanagurta*).

Miller & Robbins (27) have published an extensive study on the chemical composition and vitamin content of the opihi, the Hawaiian limpet, an important native foodstuff especially in older days. A great number of fresh and dried sea fish have been analysed by Patwardham *et al.* (29) for protein, fat, calcium, phosphorus, iron, and, in some cases, for vitamin A. The results show that these fish constitute a cheap source of animal proteins and essential minerals such as calcium, phosphorus, and iron.

Moore advocates the use of cheap vitaminized natural oils in vitamin A deficiency instead of red palm oil (28). Cluver has published a valuable paper on nutrition in South Africa (30).

FOOD CONSTITUENTS

In the tropical dietary protein and fat deserve special attention as principal constituents: protein, not only because little or no animal protein appears in the diet of many inhabitants of the tropics and the Far East, but also because the total protein supply is often very low, i.e. when judged by the standards of the nutrition worker in temperate climates. It should not be concluded, however, that all inhabitants of the tropics and the Far East live on a vegetarian or protein-deficient diet.

The same remarks apply to fat. The total quantity of fat, usually almost exclusively of vegetable origin, in the daily menu of many tropical inhabitants is very low, compared to the usual standards in Europe and North America (see also p. 406).

It is obviously of great importance that more attention be paid to the proteins and fats consumed as the supply of protein and fat becomes less varied, more vegetarian and less ample. Biological and supplementary values and the amino acid composition of proteins, as

well as analysis of the fats (linoleic and linolenic acid, sterols, vitamin A) of tropical foodstuffs are clearly very desirable.

*Proteins.*⁵—It appears from experiments with rats (31) that it is desirable that protein and carbohydrate be consumed together rather than separately. According to Harris *et al.* (32) the protein requirement of adults is much lower than that of younger persons. A second publication by Almquist has appeared on his chemical estimation of quality in animal protein concentrates; this appears to give useful results (33). According to McHenry & Gavin (34) pyridoxin (vitamin B₆, adermin) together with thiamin and other vitamins, is essential for the conversion of protein into fat in the organism. Chang, who gives a very valuable analysis of cabbage leaves (*Brassica chinensis* L), which are consumed in great quantities in China (35), mentions the efficient extraction of many vegetable proteins that may be obtained with 0.05 N NaOH (36).

Basu *et al.* published investigations on the biological value of the proteins of Bengal fish (37).

Hsien Wu, Chang, *et al.* are continuing their interesting experiments on the influence of vegetarian dietaries on the life and welfare of the rat (38, 39, 40, 41). For the results the original publications must be consulted. The cause of cataract, parathyroid hypertrophy, and hypocalcemia in their rats appears to be a deficiency of vitamin D in their vegetarian diet. In connection with the vegetarian nutritional habits of the Chinese rural population an older but very interesting paper of Adolph is worth mentioning (42), in which the great practical value of this large scale, long term experiment is stressed.

According to King (43) the digestibility of proteins in watermelon and pumpkin seeds is 92 at a 10 per cent level of feeding; the biological values are 73 and 63, respectively. Both are rather important products in some parts of the Far East. In connection with their own experiment (44) Basu *et al.* discuss the significance of the S/N ratio in human urine; on a protein-free diet they find a ratio of 1:8.5 and in fasting metabolism 1:14. Nitrogen and sulphur fractions in the urine of a subject living on diets containing different amounts of rice and wheat were also determined.

Fat.—Although it remains to be seen whether a human being fed on normal, nondenatured food can contract the same sort of deficiency as rats on the fat-free diet of Burr *et al.*, it will be worth while to re-

⁵ See also "Diseases and Nutrition," p. 401, and "Requirements," p. 406.

member that according to Turpeinen and other investigators (45), arachidonic acid esters are as active as linoleic acid in prevention or cure of the fat-deficiency disease.

Carbohydrates.—A very valuable editorial on the availability of carbohydrates in the diet and factors in connection therewith appeared in the *British Medical Journal* (46).

Vitamins.—Reviews on this subject by others will be found elsewhere. The few publications mentioned here deal only with the tropics and the Far East. Although a great deal of the older knowledge of vitamins is due to the researches of investigators in the tropics and the Far East, the development of the newer knowledge and the practical application of vitamins has on the whole made more progress in other countries.

This is partly due to the fact that in these tropical regions the greatest attention has in the first place to be paid to the prevention and curing of the numerous diseases (of nutritional or other origin) which are already known to exist there; impediments are also presented by lack of equipment, difficulties of work, and lack of available time. In these regions conditions for research are less favourable than in cooler regions, where there is less poverty, less disease and undernourishment, and where the number of investigators is much greater.

Reports on the vitamin contents of the many tropical foodstuffs, often unknown elsewhere, are, of course, of great importance.

Luck gives a valuable review (47) of the advantages and disadvantages of fortification of foodstuffs; the enthusiastic supporters of fortification of rice and other principal foodstuffs with vitamins A and B for the millions of poor in the Far East will learn from this article that even in America and Britain the question is far from being a simple one.

Mar determined the vitamin-A content of the livers of some twenty-five kinds of Chinese fish (48) and Basu & Rai Sircar that of Bengal fish (49). Leong determined the nicotinic acid content of a great number of tropical foodstuffs (50) using the cyanogen bromide method of Swaminathan. A valuable review of the distribution of nicotinic acid in foodstuffs is given by Bacharach (51). Chang & Hsien Wu (52) find the vitamin D content of many kinds of cabbage leaves, which play an important part in the native diet in many regions, to be rather low.

Minerals.—Of the studies on minerals, especially calcium, phos-

phorus, and iron, only those relating directly to human diet and health in the tropics are mentioned.

Basu and others (53) come to the conclusion that (compared to western standards at least) one of the greatest defects of typical Indian dietaries is insufficiency of calcium. An experiment on seven adults concerning the utilisation of calcium in milk proved that, while some persons utilise the calcium very well, others do so to a less extent, but for this difference no special reason could be found (54).

From certain animal tests it appears that the calcium content of the food has no influence on the biological value and digestibility of protein; this may be of importance to many inhabitants of the tropics whose diet often lacks calcium (55). This agrees with the findings of Swaminathan (56) but not with those of others (57).

Whereas the phosphorus of yeast nucleic and soybean phosphatides is just as easily available for bone calcification as inorganic phosphate, vitamin D has a greater influence on the utilisation of phosphorus from phytic acid than from the former sources, although phytin phosphorus still remains less efficient (58).

Potgieter determined calcium and phosphorus balances on diets high in taro, the important native foodstuff in many regions of the Pacific (59), and concludes that both elements are well utilised. In a Japanese subject calcium balance was found at the low intake of only 0.091 gm. per day.

An important report on the relation between dental caries and diet is given by McCollum (60) in which he discusses clearly the role of sugars, starch, vitamins A and D, rickets and microorganisms. Trendley Dean *et al.* found a negative correlation between the fluorine content of drinking water and dental caries (61). East comes to the conclusion (62) that the amount of solar ultraviolet energy is related to the amount of dental decay in the teeth of children and that the incidence of caries in children is lowered when they are regularly fed vitamin D. A close correlation was found to exist by Arnold *et al.* (63) between the number of acidophilous organisms in the saliva and dental caries activity, but not between the amount of caries and other (chemical) data.

Tratman (64) is of the opinion that, in certain areas in Malaya, the low supply of minerals, especially of calcium, is compensated for in great part by the widespread habit of betel-nut chewing (a mixture of areca nuts, lime, and betel leaves). According to Tratman neither hypoplasia of the teeth enamel nor a high cereal diet is to be consid-

ered as the primary cause of dental caries; a metabolic unbalance, however, predisposes to this.

The diverging requirements of the skeleton and the teeth with respect to calcium and phosphorus are clearly discussed in a publication by Gaunt & Irving (65).

Adolph & Hsu (66) found, in their efforts to compose a cheap low-class Chinese diet with higher calcium content, that 3 gm. of bone ash per day would be sufficient as a dietary supplement, whereas soybean milk in contrast to cow's milk did not give satisfactory results.

The relative independence of dental metabolism from that of the skeleton has been proved by recent investigations in which an acute magnesium deficiency in rats reduced the magnesium content in the skeleton considerably, but did not change that of the teeth (67). According to Irving (68), however, characteristic tissue changes took place as the calcification rhythm became upset. A statistical investigation (69) proved that increased immunity to dental caries in a group of children was associated with increased fluorine intake.

It is evident that the abundant tropical rains in many mountainous regions in the tropics considerably erode and wash out the mountain slopes, as a result of which the iodine percentage of the soil may become reduced to a minimum. Goitre is indeed very prevalent in these regions and the use of iodised salt may be of considerable value. Although potassium iodide mixed with sulphite in salt briquettes is considered sufficiently stable, other methods of stabilisation also deserve attention. Such a method is that of Johnson & Frederick (70) in which the potassium iodide particles are protected by a thin film of fine calcium stearate.

According to Wilson the distribution of endemic goitre in the Punjab and in England is related to the geological distribution of fluorine and to human dental fluorosis (71). A series of publications appeared on endemic fluorosis in South India by a team of workers in Madras (72).

The zinc and cobalt content of a number of tropical foodstuffs has been determined by Morris (73).

DISEASES AND NUTRITION

It is now known with certainty that many of the diseases which occur in the tropics and the Far East are due to nutritional deficiencies (beriberi, pellagra, rickets, struma, nutritional edema, nutri-

tional anaemia, Bitot's spots, sprue, phrynoderma, angular stomatitis). In so far as these diseases are more or less clear-cut deficiencies, they will be discussed in the reviews on vitamins, minerals, etc. This also applies to epidemic dropsy in India, the cause of which is now known to be the oil of *Argemone mexicana*. Only those studies are mentioned here, however, which are of special importance for this review. There are also other diseases, for example certain forms of cancer, leprosy, tropical ulcer, in which nutrition is suspected to play a part, but in regard to which definite proof is not yet available.

A good review on the cutaneous manifestations due to vitamin deficiency in the tropics is given by Sequeira for Africa (74), also by Neave Kingsbury *et al.* (75) for Malays and Tamils living in Malaya. Fitzgerald Moore (76) writes about the difficulty which may arise if night blindness as well as phrynoderma are considered to be early manifestations of a vitamin A deficiency.

Ahmad & Seshan (77) found that the average vitamin A reserve of Indian working-class people was between 7 and 10 $\mu\text{g.}$ per gm. of liver and for the middle class about 25 $\mu\text{g.}$ These values are indeed low. Ralli *et al.* (78) determined the vitamin A and carotene content of the livers of 116 subjects. In the patients with cirrhosis of the liver and in those with acute infections a striking decrease in the vitamin A and carotene of the liver was found.

Riddle, Spies & Hudson (79) conclude from their observations on some 150 patients suffering from deficiency diseases (clinical pellagra, riboflavin deficiency, and beriberi) that there is a relation between these diseases and the resistance against (or presence of) infections by *Staphylococcus aureus* and *Streptococcus haemolyticus*.

Fehily published an interesting treatise (80) on infantile beriberi in Hongkong, and on the negative peroxidase reaction and the methyl glyoxal content in the milk of women suffering from avitaminosis. Hou (81, 82, 83) made a special study of riboflavin deficiency amongst Chinese. Kochhar (84) concludes from nicotinic acid determinations in blood that these are probably of little value in the diagnosis of pellagra. Naganna *et al.* (85) determined the urinary excretion of nicotinic acid in a number of Indian pellagrins. The relation between pellagra and nicotinic acid has been excellently described in a review by Elvehjem (86). Manson-Bahr recommends the treatment of sprue (87) with riboflavin and assumes that tropical sprue represents the fully developed picture of small intestine deficiency and presumably is due to previous damage to the intestinal mucosa. Although in most

tropical countries vegetables and fruit are obtainable in sufficient quantity to supply ascorbic acid there are still periods and regions where one would expect avitaminosis-C. However, actual avitaminosis-C is found less often than one would expect. This is perhaps due to the fact proved by recent investigations of Lund & Crandon (88) that the ascorbic acid level in the blood drops rather quickly (in about six weeks), but that it may take much longer for scurvy to develop fully (five to six months). In most tropical regions, however, leafy vegetables and fruit are always available for most of the year. A very good review of ascorbic acid and its effect on nutrition is given by Fox & Dangerfield (89); the paper deals extensively with the situation amongst the native mine labourers in South Africa. Fox also presents a valuable discussion (90) of previous experiments by Crandon, Lund & Dill (cf. 88) as compared with his own. Liu (91) writes about osteomalacia as a nutritional disease in China. The observations by Sharpless *et al.* on the goitre-producing (for rats) factor from soybeans and the inhibition of this factor by iodine are confirmed by Wilgus *et al.* (92). They could not extract this factor, however, with fat solvents.

Liver cirrhosis and cancer.—In a survey by Rich & Hamilton (93) the possibility is discussed that liver cirrhosis may be the result of a dietary deficiency. Rich & Hamilton succeeded in producing liver cirrhosis in rabbits by feeding certain highly deficient diets; an unknown substance in yeast was found to be protective. Other investigators succeeded in inducing cirrhosis by feeding rats (94) on a deficient diet and giving them solutions of alcohol to drink instead of water. These diets had a low casein and cystine content and perhaps other deficiencies also.

P. György and others (95) found they could produce liver cirrhosis as a regular symptom in rats through a simple diet containing 10 per cent casein, 64 per cent sucrose, 20 per cent lard, 2 per cent cod liver oil, and 4 per cent salt mixture as well as sufficient quantities of thiamin, riboflavin, pyridoxin, and pantothenic acid. Cystine strongly accentuated the symptoms; choline had an inhibitory influence, but only when cystine was present. Yeast also had a beneficial effect. Analogous results were obtained by Blumberg & McCollum (96) who used diets as rich in fat and as deficient in protein as that employed by György *et al.*; cystine had no influence, and methionine had only a slight effect, whereas choline acted as a protective agent. Two other investigators (97) could not produce liver cirrhosis in rats by

means of a diet which lacked the vitamin B complex. An explanation of this seeming contradiction may possibly be found in the observations of György *et al.* (95) who found that casein like choline acts to a certain extent antagonistically.

A noticeable fact is that stomach carcinoma very rarely occurs among the Javanese; on the contrary, it is very prevalent among the Chinese living amongst them. Holleman, Koolhaas & Nijholt (98) point to the fact that the Chinese menu is of an entirely different composition and that according to qualitative and quantitative studies it usually contains much more animal protein and fat than the Javanese menu. They assume quantitative differences in the cholesterol metabolism of the two groups, which may have an important relation to the problem as compared with the interesting, but not sufficiently confirmed experiments of Roffo on the influence of irradiated and heated fats and sterols on the occurrence of stomach carcinoma in rats.

With reference to the fact that primary liver carcinoma is found so frequently amongst rice consumers the numerous experiments of Japanese investigators (Kinosita, Yoshida, etc.) are considered very important; in this work liver carcinoma was produced in rats by mixing the food with butter-yellow (dimethylaminoazobenzol) or O-aminoazobenzol. It was easy of accomplishment in polished rice diets but very difficult or even impossible in the case of husked rice, millet, (99), wheat, dried liver, yeast, etc. Some Japanese investigators (quoted in 99) assume that the unsaponifiable part of the "rice oil" and especially the crystalline sterol fraction of it acts protectively.

Sugiura & Rhoads (100) found that substances extracted by ether from rice bran and yeast give a very high degree of protection. Bonne *et al.* (101), on the contrary, state that this property should be accredited to the extracted rice bran. However, the extraction technique differed in the two cases.

Mori found that cystine has not a protective action (102); he used, however, a very low cystine concentration (1 gm. per kg. food). Bonne *et al.* found methionine to have no influence (101). In a recent publication (103) a group of investigators found that a very large quantity of riboflavin (5 mg. per rat per day) together with one or more unknown factors in vitamin-free casein will prevent this liver carcinoma. Wheat germ oil does not act protectively (104). Taking the previously mentioned cirrhosis experiments as a starting point György, Poling & Goldblatt (105) found that cystine and choline together, but not separately, could prevent butter-yellow carcinoma.

The possibility that rice possesses special carcinogenic properties in these butter-yellow carcinoma experiments is not wholly excluded. Rhoads & Kensler report an interesting finding (106) on the influence of butter-yellow and its degradation products on the enzyme systems in the liver.

Leprosy.—Oberdoerffer & Collier, basing their studies on various previous publications by Clark, claim that consumption of the roots of *Colocasia esculenta* (taro) may make the human organism susceptible to leprosy infection; these roots contain sapotoxins that have a deleterious effect on the suprarenal glands. Collier (107) fed a number of monkeys on *Colocasia* roots and afterwards inoculated the animals with leprosy material. He states that in some instances lesions developed which closely resembled those of human leprosy; bacteriological examination also revealed a similarity.

Nutritional edema and hypoproteinemia.—As the diet in the tropics and the Far East often lacks protein, all papers dealing with protein nutrition, plasma protein, etc. are important to this review. With regard to the occurrence of nutritional edema resulting from hypoproteinemia, it is very essential to have a thorough knowledge of the proteins of the plasma. An excellent review on this subject has been published by Madden & Whipple (108). A second paper (109) deals with the influence of the degree of hypoproteinemia and of certain amino acids on the production of blood plasma. Van Veen compared some six methods for the determination of total protein and of the albumin-globulin ratio in the plasma and sera of patients suffering from nutritional edema. He came to the conclusion that for the routine examination of many samples Robertson's refraction method with an Abbe refractometer is most suitable (110).

"Buaki" is a curious disease, common to manioc eaters in the Congo, in which edema with skin lesions and anaemia occurs. It is probably due to a badly balanced diet containing little protein (111).

Of importance to the occurrence of edema, although not to the preliminary hypoproteinemia, may be the existence of antidiuretic substances in the urine of patients suffering from Bright's disease, Cushing's syndrome, and diabetes insipidus, referred to in the experiments of Robinson & Farr (112).

According to a group of workers (113) the quantity of nitrogen excreted in the urine is lower if the food contains less inorganic base, while on the contrary the total nitrogen in the blood is higher. This observation may be of importance in relation to diets which lack pro-

tein and minerals. In the case of dogs with hypoalbuminemia which were fed on a diet lacking in protein, the liver protein became reduced and at the same time the water content of the liver became higher (114). This supports the impression that the liver acts as a reservoir for the blood albumin.

It is known that the basal metabolic rate is considerably reduced in many edematous conditions. According to Moschcowitz (115) this may be explained by the fact that the edema fluid acts to some extent as an isolating medium by which heat conduction and convection are prevented.

REQUIREMENTS AND STANDARDS

It has been mentioned previously that the dietary protein and fat in the tropics are often almost exclusively of vegetarian origin and often very low, as compared with Western standards. Yet numerous investigations into the diets of many regions of the tropics and the Far East have proved that these diets do not always induce a state of malnutrition and ill-health. This leads us to wonder whether the customary (Western) standards and requirements are not sometimes too high, or whether they are applicable to all races and climates (even after considering body weights, etc.). This query applies to the so-called "optimal" as well as to the "minimum adequate" requirements which, no matter how difficult to define and to determine, are easily understood.

This question is dealt with in the reports of the League of Nations Intergovernmental Conference on Rural Hygiene (1937), as well as in the reports of the League of Nations Technical Commission on Nutrition of 1937 and August 1938. Whereas in countries blessed with greater material wealth, one can afford to speak of "optimal" nutrition and "optimum" requirements, the nutrition worker in the tropics and the Far East has to be content with nutrition conditions, menus and requirements which are "reasonably satisfactory." One meets here, of course, with as much difficulty in making a distinction between minimal adequate or inadequate, as elsewhere in determining real optimal values; but for the nutrition worker in the tropics the former is of greater practical value. These considerations are also to be found in a publication by Bacharach & Drummond (116) who make a distinction between "optimal" and "marginal" diets.

Raymond (117) also recently emphasised this and reviewed the

subject as it applies to conditions in East Africa. In Australia a scale of family coefficients has been drawn up (118) derived from the food purchased by the household and evolved by a form of statistical treatment. This scale agrees with that of Lusk. Recently a paper appeared on nutrition standards in the Philippines (119). Van Veen has published (120) cheap ration schedules based on the average protein and fat consumption found in the average Javanese in good nutritional condition. These may not appeal to the nutrition worker in temperate climates but have been used and found satisfactory in jails, etc., in Java, for years. Van Veen states (121) that in the most prosperous region in Java the total fat consumption per capita per day (i.e., for a per capita body weight of about 33 kg.) amounts of 5 to 12 gm. per adult of 50 kg. weight with a consumption of 2,600 calories. He is therefore of the opinion that one should not speak about a requirement-norm for fat, but only of a habit-norm, and that the danger of linolenic acid deficiency (should this occur in man) is very small, since fats from rice and maize and the principal nonrefined fats in Javanese food (cocoanut fat and peanut oil) contain a sufficiency of these fatty acids. Meyers is of the opinion that adaptation to a low thiamin intake is also possible (122).

Opinions on the calcium requirements of different individuals and races still vary considerably. Basu and others published a third paper on protein, calcium, and phosphorus metabolism on typical native diets (53) and found that the protein and phosphorus supply were adequate but that one of the greatest defects was insufficiency of calcium.

Harris *et al.* (123) come to the conclusion that there is no evidence that the greater part of the English population is suffering from the consequences of calcium insufficiency. They are of the opinion that it can be affirmed with a high degree of certainty that the calcium intake under the present dietary regime is ample, and even that very many English people consume more of this mineral than is necessary. McCance, however, does not agree with this (124). See also the editorial article in *Lancet* (125). Others are even of the opinion that Sherman's calcium standards are too low (126). Steggerda & Mitchell (127) have given a valuable review on this subject. It is worth while in connection herewith to refer to a previous publication by Nicholls & Nimalasuriya (128), in which an adaptation to a lower calcium supply is discussed, for example by a decreased excretion in the urine. See also (54) and (59).

Basu & Malakar (129) carried out some twenty magnesium metab-

olism experiments on three adults who lived on a typical native cereal diet. The maintenance requirement for an adult was estimated at approximately 0.43 gm. per day. Shukers *et al.* (130) are of the opinion that infants from two to six months of age can be kept in magnesium balance with 10 to 20 mg. per kg. of body weight per day. They did not find a simple relation between magnesium and calcium retention, fecal loss of phosphorus and magnesium, and vitamin D level and magnesium retention.

In an extensive study of iron balance in thirty-five experiments carried out on four normal pre-school children, Porter (131) came to the conclusion that there was no reason to worry about the alleged necessity for an especially high iron supply in the diet of these children. The food of the children studied supplied an average of 0.31 mg. iron per kg. of body weight of which an average of 22 per cent was retained.

Leverton is of the opinion (132) that there is no need for emphasis on iron intakes exceeding 6.5 mg. daily in the diet of normal young American women. Basu & Malakar (133) came to the conclusion that the iron requirement in two cases studied was 9.4 mg.—which agreed with the statement by Sherman—and that the so-called available iron (determined by the dipyridyl method) has but little effect on iron retention.

Heat production.—Scott, MacGregor & Loh (134) made a comparative study of the basal metabolic rate in the different races found in Singapore. This town is ideal for the purpose since so many different races have been living there for a great number of years under the same climatic conditions. In all the groups which have been studied, the basal metabolic rates were lower than the corresponding values in the temperate zones. Two groups are to be distinguished, one which falls about 5 per cent, and the other about 10 per cent, below the normal Du Bois standard. Racial influences exist side by side with separate climatic influences. Niyogi, Patwardhan *et al.* (135) come to the conclusion that since dietetic, climatic, and other factors do not lower the basal metabolic rate in their experiments this is strong though indirect evidence in favour of the racial characteristic being the principal cause of the low metabolic rates observed in their subjects.

Radsma (136) came to the conclusion from his experiments that the low basal metabolic rates observed in the tropics are not exclusively due to this high environmental temperature. He found that even under

the most favourable circumstances the metabolic rate in a tropical environment does not become reduced to the extent that it does when the test person rests in cooler air-conditioned surroundings (137).

Radsma & Vos (138) state that rats reared for three generations in an air-conditioned room showed a considerable increase in body weight, which has, however, not yet been proved to be due to the low temperature of the environment.

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CALCIUM AND PHOSPHORUS METABOLISM: CLINICAL ASPECTS¹

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Mineral metabolism was last reviewed in the *Annual Reviews* in 1939 (1), largely as an extension to an exhaustive review published in 1935 (2). Since then, other reviews of parts (3, 4, 5) and the whole (6) of the field have appeared.

The present authors have taken advantage of the liberal policy of the editors to choose those sections of the field, and those papers within each section, which in their opinion contribute most to the subject. Reference is made to work published prior to 1939, principally through the above reviews, in order to give the background of recent contributions.

Dietary requirements.—A great deal of interest in the optimum human dietary, brought about chiefly by the progress in vitamin research, is reflected in a renewed endeavor to determine the human requirements for calcium and phosphorus, particularly since it has been pointed out (7) that the nutritive values of American diets of different income groups differ most with respect to their content of calcium and vitamins A, C, and B₂. Several accepted standards and definitions have been reviewed and sharply criticized (8, 9, 10), and some new concepts have made their appearance. The accepted requirement (11) for dietary calcium has been challenged (12) on statistical grounds and because of the failure of many investigators to recognize the criteria necessary to determine true maintenance requirements (13, 14, 15).

In the following discussion emphasis is placed upon calcium, since phosphorus is found in adequate amounts in most adult diets, particularly so if calcium is optimal.

In order to evaluate the efficiency with which milk calcium can be utilized (absorbed) and to set individual requirements in the light of this utilization, it is necessary to determine the critical level of dietary calcium above which no increase in retention (deposition) is found (14, 15, 16), and to presaturate the individual subjects to remove any

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existing calcium deficiency (14). [In infants no maximal retention has been found, and the net retention of calcium and phosphorus is directly related to the intake (4).] Then, by measuring the increase in retention at two levels, both of which are below this critical level, and dividing by the increase in intake, the fraction of dietary calcium that the body can utilize is obtained (15). If one merely divides the total retention by the total intake, a figure for percentage retention is obtained which can be less, but not greater, than the percentage utilized, because it will include the "maintenance" requirement ("endogenous," urinary loss). In growing children the maintenance requirement approaches zero (15, 17) so that retention becomes nearly equal to utilization (absorption).

With these concepts, the maximum retention of calcium by pre-school girls (14) and boys (15) is found to average 7.5 mg. per kg. per day, less than half the usually quoted figure (11). This retention is obtained on a dietary intake of 32 to 44 mg. calcium per kg. (about 600 mg. per day) from one pint of milk plus the usual vegetables. Worthy of note is the fact that these growing children can utilize only about 20 per cent of the ingested calcium (15, 17), from either milk or $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ (18), while the growing rat can utilize 90 to 100 per cent. Other reports indicate maximal retentions in ostensibly well-nourished children of 11 to 12 mg. calcium and 7 to 9 mg. phosphorus per kg. per day (19, 20). The observation is made that, as judged from the Todd (21) and Harvard (22) skeletal standards, 80 per cent of children are undernourished with respect to calcium (20), which may account for the higher retentions usually quoted.

Adults are found to utilize about 24 per cent (23) and 29 per cent (24) of ingested milk calcium or calcium gluconate, even when in negative balances on intakes of 450 to 870 mg. per day (23). Adult utilization is variable and may range up to 50 per cent (25, 26). From the average, the requirement for zero retention (balance) is set (27) at an average of 660 mg. per day, or 10.7 mg. per kg. per day. This is higher than the heretofore accepted figures of 550 (11) and 450 (28), which have been reviewed and criticized (12, 27) principally because they are based on the assumption that the human can utilize 50 to 100 per cent of ingested milk calcium. There is a maintenance requirement (loss on zero intake) of about 2.7 mg. per kg. (24); thus, in adults, utilization exceeds retention.

A study of sixty adults, at "equilibrium" on a minimum average intake of 6.5 mg. per kg., is reported (29). However, raising the

intakes to higher levels in a series of ten older male subjects resulted in large positive balances of calcium and phosphorus, presumptive evidence of a long standing calcium deficiency; this suggests that senile osteoporosis may be a simple dietary deficiency (30). Furthermore, the facts that several such subjects could be kept at zero balance on low (3 to 4 mg. per kg.) intakes and that four out of seven were osteoporotic by x-ray diagnosis leads one to conclude that balance is not the criterion of the correct requirement in adults any more than it is in children. This rests on the postulate that maximum retention of calcium is the best criterion of adequate intake. If this is so, racial adaptation to low calcium intakes (31) may not exist in fact (32) or else is really due to an increased ability to absorb calcium (33), because of some dietary peculiarity. It is of interest that a low protein diet may lead to negative calcium and phosphorus balances on an intake that is considered optimal (34).

Thus milk, regarded as the best source of calcium, is very imperfectly utilized by man. With the calcification of bones as the test object the retention of ingested calcium by growing rats (on a cereal-free low-calcium diet) was found (35, 36) to reach 60 to 100 per cent of the intake from a milk source; vegetables furnished 74 to 85 per cent of their calcium in comparison with milk (35, 37, 38, 39). This was not altered by cooking or canning. Spinach lowers the utilization of milk calcium (38), probably due to its oxalate content (40, 41). Of speculative interest in connection with the utilization of milk is the fact that only about 20 per cent of the calcium in milk is ultrafilterable (42). Calcium ion constitutes about 20 per cent of this fraction, the rest of it being in lactose and citrate complexes. The effect of lactose and citrate in enhancing the absorption of calcium is well known, and thus the small concentration of free calcium ion in milk may be of significance in decreasing the precipitation by phosphate in the intestine.

It is thus to be noted (3, 14, 15) that there is considerable difference in the percentage utilization of ingested calcium by rats and humans.² That this is largely a matter of absorption is shown by the

² It would be much less confusing if all the pertinent work reported in this review referred to one species of animal. However, most of the work dealing with calcium and phosphorus metabolism in the last few years has utilized either human beings or rats. This is particularly unfortunate since much evidence has accumulated to show that these two species differ considerably in their absorption as well as bone reactions to many factors which influence calcium metabolism.

differences in urinary excretion of calcium when other factors (see *Absorption*, p. 419) are ruled out. In rats the proportion of calcium excreted by the kidneys greatly exceeds that in man. The growth of the rat fetus and its storage of calcium and phosphorus depend upon suitable plasma calcium and phosphate concentrations in the blood of the mother; these concentrations can be maintained by dietary means (43). Raising the calcium intake of an adequate diet (tested over many generations) in rats gives a definitely higher percentage of calcium in the body, the increment amounting to 10 per cent at the most rapid growth period and 3 per cent at maturity (44). Furthermore, the general condition of the animals throughout their life span is better (45). These results indicate that an apparently optimal calcification rate can be increased (46). This is in harmony with the concept of a calcium "storehouse" in the bones (46a). Larger rats have a somewhat lower percentage of calcium than smaller ones (46), but the rate of growth does not of itself determine the rate of calcification (47, 48). Increase in phosphorus intake has also been found to increase the percentage of ash in growing rats (49). The development of rickets in growing infants and the so-called physiological loss of calcium in early human infancy also indicate the independence of growth and calcification rates (4). The apparently limitless ability of the infant to retain calcium and phosphorus (4) is also in keeping with this hypothesis (47).

Tracer studies.—The results arising from studies utilizing radioactive isotopes as tracers were reviewed in 1940 (50). These results have been of value in demonstrating the fluidity of the chemical constituents of the body and in discovering pathways and mechanisms. The fact that only a very small fraction (2 per cent) of injected radioactive phosphorus (P^{32}), as disodium hydrogen phosphate, appears in the feces within two months (51) demonstrates the small part that the blood plays in the origin of fecal phosphorus. When the same amount is given by mouth as a salt alone, 93 per cent remains in the body for over two months, while if given with food one third of it appears promptly as fecal phosphorus. Some of the absorbed P^{32} appears in the urine within the first few hours.

The uptake of P^{32} by tissues has been followed in many animals. In general, the uptake (per unit of phosphorus) is rapid and transitory in compounds of a functional nature (e.g., adenosine triphosphate), slower and more lasting in compounds of a structural nature (e.g., bone), approaching zero in the enamel of adult teeth. A rapidly grow-

ing (52), rapidly metabolizing tissue shows the greatest relative P^{32} uptake. These principles hold in the cases of tumors (53, 54, 55, 56), in the phosphorus fractions of various tissues (57, 58, 59, 60), in bone, and in teeth (61 to 66). Studies on leukemic patients treated with therapeutic amounts of P^{32} have been published (67, 68, 69).

The relative stability of diaphyseal bone with respect to epiphyseal is shown by the fact that, in rabbits, there is a 30 per cent turnover of the latter in a time (50 days) during which only 7 per cent of the phosphorus of the former is replaced (62). During this interval half the scapula is replaced. The dynamics of the entry of P^{32} into bone have led to the concept of two fractions of bone, one "labile" [cf. "functional" (70), "organic" (71)], exchanging with plasma phosphorus, the other "stable" [cf. "structural" (70), "inorganic" (71)], receiving its phosphorus only from the labile fraction (63). Calculations have been made of the amounts and rates of exchange between the fractions of various bones and teeth in the rat (63). A phosphorus-deficient diet leads to an increased P^{32} deposition in actively calcifying tissues (e.g., incisors) (65). The movement of P^{32} from dentin in permanent teeth to enamel has been found to be so slow (61, 62, 64) as to exclude any possibility of acute changes in nutrition altering the composition of the enamel. Some of the enamel P^{32} may be derived from the saliva by adsorption (66).

The first studies with radioactive calcium (lactate) again point out a species difference in the metabolism of calcium: absorption and retention in the rat were 90 per cent and 24 per cent (72), while in the mouse these figures were about 30 per cent and 20 per cent (73). About 60 per cent of intravenously injected calcium was deposited in bone in one day in mice (73, 74). These investigations show again that absorbed calcium is usually excreted nearly exclusively in the urine.

Absorption.—The absorption of calcium and phosphorus from the intestine is influenced by a great variety of factors, all of which should be controlled if reliable data are to be obtained.

Rats can absorb far more calcium (from calcium lactate) and phosphorus (from disodium hydrogen phosphate) than is retained, excreting the excess in the urine (72, 73, 75, 76). There is no evidence of any considerable secretion of plasma calcium or phosphorus into the large intestine (77); the fecal calcium and phosphorus are fairly constant (76) and have little relation to each other or to the plasma concentrations (17, 78). The net absorption of ingested cal-

cium is not affected by a diet containing 30 per cent fiber, which causes a large increase in intestinal secretions (79). Thus the efficiency of reabsorption of secreted calcium is very high in the rat.

However, other influences upon the intestinal tract of the rat have measurable effects. A large fecal loss of calcium in normal rats can be produced by laxatives (75), which may be one reason why mineral oil reduces the effectiveness of vitamin D (80, 81). The large fecal loss of calcium by rats injected with thyroxine can be largely explained by hyperperistalsis and overeating (75)—the increased urinary loss, by an increased water intake (82). (However, these are not grossly manifest in the naturally occurring hyperthyroidism of man.) There is no change in the plasma calcium during these events.

The absorption of calcium salts injected directly into the duodenum of anesthetized dogs bears no relation to size, concentration or pH of the dose, or to the serum calcium level (83). However, when solutions of calcium chloride or calcium lactate were circulated through Thiry-Vella fistulae in dogs (84), it was found that concentrated, acid solutions of calcium chloride yielded the most calcium to the body. Alkaline solutions quickly became acid to the pH characteristic of the gut section involved, and osmotic equilibrium was achieved by passage of water across the intestinal wall.

In man there is a secretion of calcium into the intestinal tract of 0.3 to 0.8 gm. per day (3), which is of the order of magnitude of the entire daily requirement. The calcium ion concentration in the gastric juice varies with pH from 2 to 4.5 mg. per 100 cc. (85), while the other secretions of the intestines range a little higher (3, 86). Thus, if the ability of the intestinal wall to absorb calcium is a limiting factor in determining net calcium absorption, factors which influence the volume (and to a lesser extent the calcium content) of intestinal juices must be considered to influence the absorption of calcium. Because the fecal excretion of injected calcium, or strontium, is negligible in comparison with the 90 to 100 per cent appearing in the urine (87, 88), the reabsorption of the secreted calcium must be nearly complete. In the same subjects, 75 to 80 per cent of the ingested calcium appears in the feces on the same total intakes, indicating that fecal calcium is practically all nonabsorbed calcium. Yet one cannot merely state that the body absorbs an amount equal to 20 to 25 per cent of the calcium in the diet because of the unknown amount of secreted calcium simultaneously absorbed. This is a complication that has received very little, if any, attention in evaluating calcium absorption in

absolute terms. It points to the fact that any dietary constituent which affects intestinal secretion (84)—and this could include water (89)—may affect the net absorption of calcium. This is of particular importance in assaying the effects of hormones (75, 82) and vitamin D, and may go a long way towards reconciling the wide dietary maintenance requirements quoted. In experiments on native Chinese, some positive balances were obtained on intakes of less than 100 mg. per day (90), which indicates a high net absorption (33). Furthermore, postprandial administration of calcium salts or acidified milk to human subjects causes a rise in serum calcium, showing that absorption into the plasma can be more rapid than removal from it and that the absorption does not depend entirely upon the serum calcium concentration (91, 92).

The effect of the excess fat in the intestines in idiopathic steatorrhea, perhaps through interference with vitamin D absorption (81), is to produce a negative calcium and phosphorus balance (93). Calcium soaps are not absorbed in the rat (94). Because a diet low in phosphorus seems desirable for patients with renal insufficiency and hypoparathyroidism (leading to phosphorus retention), aluminum hydroxide is being used in these cases as a method of precipitating the phosphorus as aluminum phosphate in the intestine (43, 95, 96). This is similar to the mechanism of "beryllium rickets," well known in the rat (2). This method offers the advantage of allowing these patients to consume their accustomed diets, which are usually high in phosphorus.

It might be expected from the above that iron also acts to reduce phosphorus absorption by precipitation in the gut, and such is the case (97, 98, 99). Furthermore, the reverse of this (high phosphorus, low iron) may lead to nutritional iron deficiency (98). In view of the huge doses of iron used in the treatment of certain anemias, this precipitation assumes a certain practical importance in nutrition.

In cereals, 50 to 80 per cent of the phosphorus is in the form of phytic acid (inositol hexaphosphoric acid ester) (100, 101), the phosphorus of which is difficultly hydrolyzable and which forms a calcium magnesium salt of low solubility (phytin). The phosphorus of this compound is not well utilized, and about 60 per cent of the compound is excreted unchanged by humans in the feces (100). In experiments with dogs (100), on a normal calcium and phosphorus intake, phytin was found to be slightly antirachitic, while phytate (sodium salt of phytic acid) was rachitogenic in sufficient amount to account for the known rachitogenic properties of cereals. This property is counter-

acted by adding calcium. Because the compound is about 60 per cent phytate and 40 per cent phytin (100), cereals are rendered nonrachitogenic by adding calcium but not by adding phosphorus. It becomes obvious that analyses of cereal-containing diets for total calcium and phosphorus are of little significance. In addition, the division of the substance between the free form (phytic acid or sodium phytate) and the calcium magnesium salt (phytin) may account for the confusing results recorded in the literature regarding the metabolism of these compounds.

Added phytin has been shown to be antirachitic in rats (102), and added calcium (to as high as a calcium/phosphorus ratio of 2) will prevent rickets on a cereal diet (103). Using body weight and percentage of bone ash as the criteria, it is shown that these animals can utilize some of the phosphorus from phytate, and this utilization is improved a great deal by vitamin D (104). It is never as well utilized as inorganic phosphorus, however. The addition of calcium, particularly as the chloride, above a ratio of 1:1 markedly diminishes this utilization (105). Calcium carbonate and calcium phytate are equally good sources of calcium for the rat (106). These results are in harmony with those reported for dogs and humans above, if one makes allowance for the better utilization of calcium by the rat. In addition, the utilization of phytate phosphorus by rats seems to depend a great deal upon other constituents of the diet and may be related to intestinal fermentation processes.

Citrates, particularly potassium citrate plus citric acid, added to rachitogenic diets produce an increased bone ash in rats (107) and thus protect the animals from rickets. Since vitamin C is not a factor (36, 108), the improved calcium assimilation caused by orange juice (109) may be ascribed to its citrate content. The beneficial effects of citrates on calcification in rats on cereal diets is probably due to the removal of calcium from phytin by citrate, for citrate improves neither a low-calcium, high-phosphorus diet, nor a high-calcium, low-phosphorus diet that does not contain phytin (110).

Whether it is due to complex formation with calcium or to lactic acid produced by intestinal fermentation, lactose is known to increase calcium absorption in rats (111) and in infants (4). In children, on an intake of 500 mg. calcium per day (as CaHPO_4), the addition of 36 gm. lactose gives an average increase in retention of 33 per cent (112), which can be directly attributed to increased net absorption.

In connection with the explanations of the effects of citrates and

lactose on calcium absorption, it should be noted that a decreased intestinal pH and increased tissue pH favor absorption and retention of calcium, respectively (3), while the converse is equally true. A substance which will do both (e.g., citric acid, which also reduces the effective calcium ion concentration) should give the largest retention.

Calcium and phosphorus in the plasma.—The concentrations of calcium ion and inorganic phosphate (chiefly $\text{HPO}_4^{=}$) in the blood plasma are of importance for the maintenance of normal neuromuscular irritability and probably for the deposition of bone salts. Abnormal levels may be manifested in rickets, parathyroid dysfunction, renal disease, and other conditions which modify the utilization and excretion of calcium and phosphorus. It is in blood and bone that calcium and phosphorus manifest their interdependence most strongly, particularly through the calcium ion and $\text{PO}_4^{=}$ concentrations. Furthermore, calcium ion and calcium proteinate have a fluid relationship which can be approximately represented by a simple Mass Law equation (2, 3).

While these concentrations are important for the deposition of bone salts, the intermediation of cells at the site of calcification is still a necessary condition for the initiation and reversal of deposition of the salts and the translocation that takes place in growth (2, 3). Substances which act upon the cells of bone may alter $[\text{Ca}^{++}]$ and $[\text{PO}_4^{=}]$ in the plasma. Thus, vitamin D in sufficient dosage will raise the plasma calcium of rachitic children and in cases of tetany due to low plasma calcium it may do this sufficiently to relieve the symptoms (113, 115). Plasma is normally not supersaturated with respect to calcium ion and $\text{PO}_4^{=}$ (114).

Physiological alteration in the normal concentrations of calcium and phosphate in the serum is found in pregnant women where, in spite of a high intake and a positive calcium balance, the calcium concentration may drop to 8.5 mg. per 100 cc. (116, 117). Infants have a slightly elevated calcium (118) but a definitely elevated inorganic phosphorus concentration of about 6 mg. per 100 cc. (118, 119). One may visualize a relationship between these facts and (a) the marked ability of the infant to retain ingested calcium (4), (b) the relative frequency of "renal rickets" in children, with secondary hyperparathyroidism, and (c) the occurrence of renal insufficiency in children, which may lead to phosphorus retention (95, 120).

Alkalosis produced experimentally in cats causes a decrease in serum calcium from 10.9 to 8.0 mg. per 100 cc., a slight elevation of

itself was deficient only in calcium. Serum calcium dropped to 4 to 6 mg. per 100 cc., without tetany, while serum phosphorus was unchanged (122). The basal metabolism is increased, and the efficiency of utilization of foodstuffs is decreased; this occurs in magnesium deficiency as well (150). This latter effect may be due to increased specific dynamic action because of accelerated tissue destruction, or to impairment of oxidative mechanisms, thus resulting in incomplete oxidations (151). Calcium stimulates the succinoxidase activity of rat tissues (152).

Severe phosphorus deficiency (with or without vitamin D) in the rat also leads to extreme skeletal rarefaction with a urinary loss of calcium; this ceases in about five weeks when nitrogen loss sets in, thus indicating soft tissue destruction (76). No pathological changes, save those due to inanition, are found (153). With similar, less deficient diets the addition of phosphorus gives completely normal growth and bone composition, while vitamin D promotes partway normal bone composition but lessened growth (154). Experimental rickets in the rat is more of a phosphorus deficiency than a vitamin deficiency.³ Less than 1 per cent of the ingested phosphorus appears in the urine in three days (71); it is preferentially deposited in the actively calcifying tissues (65). It has often been pointed out that the usual rachitogenic rat diet [Ration 2965 (155)] has multiple deficiencies, and it is not surprising to find that it will not support normal growth and development even after the addition of vitamin D (156). The vitamin will not by itself produce a normal union of fractured bone in rats on a rachitogenic (low phosphorus) diet; phosphorus is necessary (157).

In the human, dietary calcium and phosphorus deficiencies may result in rickets or osteomalacia. The latter disease is primarily a

³ Rickets (failure of epiphyseal calcification due to avitaminosis D) is generally found in conjunction with dietary mineral deficiencies, and in the rat this is always the case. Any procedure which produces a net deposition of bone will cure rickets, but it is possible in both rat and man to cure rickets (i.e., produce epiphyseal calcification) with vitamin D without increasing, even sometimes decreasing, the percentage of bone ash in the skeleton as a whole. It is not correct to judge the extent of rickets by the general demineralization of the bone, for this would assume that rickets and osteoporosis were the same disease. Demineralization of bone cannot be considered rickets unless vitamin D is a factor in the origin and development of the disease; in this sense, "renal rickets" is a misnomer.

mineral deficiency, due in part to avitaminosis D and responding completely to therapy with calcium, phosphorus, and vitamin D (90, 158, 159, 160). Calcium and phosphorus are then retained in a 2:1 ratio (159), the large fecal losses of calcium are prevented, and calcium appears in the urine (158).

That disuse of a limb (atrophy of disuse) produces osteoporosis (resorption of bone) has been confirmed (161). The loss of calcium in the urine and the elevated plasma calcium concentration simulate hyperparathyroidism, and renal damage may result.

Hypertrophy of the parathyroids of the rat in response to rickets (162) has again been shown to occur only in the low calcium type which results in lowered serum calcium and elevated inorganic phosphorus concentrations (163). Altered concentrations like these are found in "renal rickets" with acidosis (120) and present a picture of calcium deficiency and phosphorus retention. Sodium citrate, which supplies base, helps materially to reverse this loss of calcium. If a urine is excreted that draws bone calcium for use as a base, a secondary hyperparathyroidism may produce a low serum phosphate concentration (164).

Teeth.—Low calcium rickets in the guinea pig produces typical changes in bones and teeth (165). In older animals the lack of growth obscures the skeletal symptoms, but not those in the teeth. This is the reverse of the situation in the rat, where calcium and phosphorus deficiencies produce greater defects in bone than in teeth (39). However, this situation is dependent to a large degree on the calcium/phosphorus ratio in the diet: ratios over 0.5 progressively favor tooth formation while interfering with skeletal deposition (166). The teeth of rats deprived of parathyroid tissue at birth fail to develop (167), but viosterol or aluminum acetate enables pregnant parathyroidectomized rats to maintain normal calcium and phosphorus concentrations (43). Tracer work has shown that the molars of rats and the dentin of dog teeth (64) accumulate plasma P^{32} at a slower rate than diaphyseal bone (63) and exchange of P^{32} between dentin and enamel is too slow to be influenced by nutritional alterations after the teeth are well formed (61, 62). P^{32} is deposited in fully erupted tooth enamel (in dogs, cats, and monkeys) partly from the pulp and dentin and partly by adsorption from saliva (66). It has been shown that the incubation of teeth with saliva and refined sugar or flour *in vitro* results in a decalcification which can be prevented or even slightly reversed by calcium salts with or without phosphate (168). The sali-

vas of caries-free and caries-active individuals, however, have about the same rates of flow and concentrations of calcium and phosphorus (6.3 and 15.3 mg. per 100 cc.) (86).

Caries.—A summary of findings and conclusions on the cause and control of dental caries, published by an advisory committee of the American Dental Association (169), gives a picture of confusion and contradiction (170). Although no correlation between the caries incidence and the diets of Eskimo children could be found (171), and in spite of the finding of nearly perfect teeth in children from mothers with chronic osteomalacia and other deficiency diseases (172, 173), the belief persists that the state of nutrition is an important factor somewhere in the etiology of caries (170, 174). The extreme demineralization of bone seen in hyperparathyroidism does not seem to affect the teeth (175), although occasional exceptions are reported (138, 176).

Several recent surveys have shed some light on the subject. The best teeth in a group of English children seemed to be those formed the earliest (lower incisors and canines), and a correlation was found between dietary history and quality of teeth (177). In a similar survey in this country, decreased incidence of caries in children was correlated with dietary quality and absence of pathological disturbances, while increased incidence was correlated with past rickets (178). A study of the calcification patterns of one thousand teeth (179) and of the case histories of their previous owners led to the conclusion that there are certain periods in the growth of the infant during which the formation of enamel and dentin is acutely dependent upon external metabolism. These critical periods are at the ages of 10 months, and $2\frac{1}{2}$ and 5 years, with a less critical one between 2 weeks and 10 months. Between 10 months and $2\frac{1}{2}$ years and after the fifth year there is a relative immunity of the teeth to metabolic upsets; the first decade is far more important than the following ones as far as tooth calcification is concerned (180).

Thus there is general agreement that chronic dietary deficiencies during the periods of tooth formation and rapid growth may give rise to persisting hypoplastic defects (170). Because the human tooth grows so slowly later in life it is not surprising that acute disturbances in this period have so little structural effect. Any persisting surface defects are, of course, only the first part of the story; they but constitute a predisposing factor to the later known effects (181) of diet and oral bacteria. These factors act upon the structure handed down

from an earlier, more susceptible period.⁴ Through a dual mechanism of this kind the apparently conflicting mass of data on the relation of metabolism to dental caries may be reconciled (181).

Effects of parathyroid hormone and vitamin D.—Hyperparathyroidism due to adenoma usually presents a picture of elevated serum calcium and depressed inorganic phosphorus concentrations (182, 183). The increased urinary calcium which results may give rise to stones (183) and renal failure; these symptoms are also found in the osteoporosis resulting from disuse, which thus simulates hyperparathyroidism (161). Resorption of bone but not of teeth is marked (175).

Parathyroidectomized rats show a much greater desire for calcium lactate solution than for water and a lessened desire for phosphate (184, 185); a high-calcium, low-phosphorus diet will maintain the plasma concentrations of calcium and phosphorus (186) as will also viosterol (115, 187) or aluminum acetate (43).

The applications of these findings to human hypoparathyroidism are many. Aluminum hydroxide or the acetate has been used (96, 188) to restore the plasma concentrations. Large doses of A. T. 10 and calciferol⁵ exert similar effects, principally by increasing the urinary excretion of phosphorus and raising the plasma calcium concentration, which have made them useful therapeutic agents (81, 115, 189, 190, 191).

In keeping with these facts, A. T. 10 in rats is far more antirachitic on a low-calcium, high-phosphorus rachitogenic diet than in the usual high-calcium, low-phosphorus variety (192, 193). Since healing of rickets may take place by decalcification of the bone shaft⁸ (110, 154), the increase in the percentage of bone ash is used to demonstrate that A. T. 10, in large doses, does have a small effect upon calcium deposition (192, 194). The fact that vitamin D in minimal doses by mouth or by direct irradiation restores positive balances of calcium and phosphorus in cases of osteomalacia again points to the similarity between this disease in adults and rickets in children (90, 158, 159, 160).

Modes of action of irradiated sterols and parathyroid hormone.—Irradiated ergosterol or viosterol preparations are about 50 per cent

⁴ It is of interest to note that three of the factors which may affect the early development of teeth [calcium, ascorbic acid, and vitamin A (181)] are among the dietary constituents most often missing from the diets of low-income groups (7).

⁵ A. T. 10 = dihydrotachysterol.

calciferol (vitamin D₂), the antirachitic principle; some of the remainder is tachysterol which has the property, as do dihydrotachysterol (A. T. 10), calciferol, and many other sterols, of raising the calcium concentration in the plasma (115). The amounts of calciferol (or of vitamin D₃) needed to prevent or cure rickets (*ca.* 25 μ g. per day = 1000 I.U.), by bringing about epiphyseal calcification, are much smaller than those which raise and maintain the serum calcium concentration (*ca.* 4 mg. per day). A. T. 10 is twice as efficient in this direction (*ca.* 2 mg. per day) while possessing only slight antirachitic properties (*ca.* 150 I.U. per mg.) (193). There are thus two distinct end effects exerted by the available preparations of vitamin D upon calcium and phosphorus metabolism, one of which is the deposition of bone at the provisional (epiphyseal) zone of calcification, the other of which is the dissolution of bone. Both actions increase the need of the body for calcium, although the first action (antirachitic) effects the greatest retention of absorbed calcium by depositing it in bone while the second (hypercalcemic) can cause large losses of absorbed calcium in the urine (195). Phosphate excretion decreases in the first instance, as phosphate is needed for bone, while hypercalcemic doses cause the reverse. The action of A. T. 10 should be identical solely with that of the hypercalcemic principle (115) in vitamin D; thus it raises the phosphorus excretion more and increases calcium retention and absorption less than vitamin D. Parathyroid hormone, being free from any possible contamination with antirachitic material (115), raises phosphorus excretion still more and does not increase calcium retention.

Viewed in this manner (115), it is possible to reconcile conflicting theories regarding the action of these substances. One of these theories (81, 195, 196, 197) regards the principal actions of all three materials as being upon calcium absorption and phosphorus excretion; the former decreases to zero, and the latter increases steadily as one goes down the series: vitamin D, A. T. 10, and parathyroid hormone. In an individual not suffering from rickets, vitamin D and A. T. 10 should have, and do have (195), similar actions since the antirachitic principle does not contribute to the net effect. In rachitic subjects there is a large drop in fecal calcium and a subsequent rise in urinary calcium (90, 158, 159, 195) when calciferol is given; this results in an increase in net absorption, which is not necessarily due to a direct effect on the intestine. Small doses of the vitamin raise the serum phosphorus while urinary phosphorus falls (197, 198), but larger

doses effect a rise in urinary phosphorus (93, 195, 196). Large doses maintain serum calcium and phosphate concentrations in pregnant parathyroidectomized rats (43). A. T. 10 in comparably large doses increases urinary phosphorus, lowers the serum phosphate concentration, and raises that of serum calcium just as calciferol does (115, 190, 191, 197, 199). The small effect of A. T. 10 upon calcium retention (192) may be due to traces of vitamin D (115). Parathyroid hormone has the same effects as A. T. 10 (195, 196, 197), except that no increase in calcium retention is found.

There is difficulty in reconciling the actions of vitamin D and parathyroid hormone upon phosphorus excretion with the theory that the primary action of each is on bone. This, however, is not necessary since each has also a direct effect upon the excretion of phosphorus apparently dissociated from the effects of an alteration in serum calcium concentration. Vitamin D raises the maximum rate of phosphorus reabsorption by the tubules of dogs; parathyroid hormone reduces it (198, 200). These effects are of sufficient magnitude to account for the increase or decrease in serum phosphate concentration which is found (195, 196, 197). Furthermore, this accounts for the seeming paradox (188) that the dissolution of bone produced by the hormone can result in a lowered phosphorus level.

Of interest in this connection is the interrelation of pH and plasma phosphorus. Acidosis lowers plasma phosphorus because of a decrease in reabsorption by the tubules (200); the increased urinary phosphorus comes, in part at least, from hydrolysis of diphosphoglycerate in the red cells (133, 135) as well as from the bones. Cases of rickets^a which display a lowered serum pH (acidosis) and which respond to base are known (164), while alkalosis has recently been associated again with a decrease in serum calcium and an increase in serum inorganic phosphorus. The resynthesis of diphosphoglycerate under the influence of vitamin D has been attributed to a simple Mass Law effect due to the rise of serum inorganic phosphorus (133), but it may be of significance that the inorganic phosphorus of the red cells falls below the pretreatment value while the diphosphoglycerate is increasing. This must be due either to a direct speeding up of the resynthesis by vitamin D or to an increase in pH caused by the vitamin, since the synthesis of diphosphoglycerate is favored by increased pH (135). The report of two cases of infantile rickets in which marked healing was produced by sodium citrate in the absence of vitamin D (201), and some of the cases mentioned above, indicates that rickets may

involve not only calcium, inorganic phosphorus, and vitamin D, but also pH, base, organic phosphorus, and kidney function. To this list complex hormonal actions may well be added.

Direct evidence that calciferol acts upon bone cells is afforded by the observation that bone (202) and trichina cysts (203), when stimulated by the vitamin *in vivo*, will calcify more rapidly, or at lower calcium and phosphate concentrations, *in vitro* than those not so stimulated.

Rats with rickets produced by high-calcium, low-phosphorus diets show epiphyseal calcification when calciferol is given. Here an increase of the plethora of calcium could hardly bring about healing (204, 205), yet the degree of healing is better than in rats suffering from low-calcium, high-phosphorus rickets, where increased calcium absorption would be a help (193). The healing of low-phosphorus rickets can be brought about without added phosphorus (204) by decalcification of the bone shaft³ (192) and by starvation and tissue destruction (205); with vitamin D there is only a slight increase in phosphorus absorption and no change in the very low urinary phosphorus excretion (71). These results indicate an effect upon phosphorus deposition, which, of course, is also upon calcium deposition. It requires nearly three days for vitamin D, in small doses and with practically no added phosphorus, to cause increased metaphyseal deposition of phosphorus, while it has no effect at all upon the diaphyseal exchange (204), and this deposition is simultaneous with healing of rickets as shown by the line test. Thus a considerable time is required for the bone cells to deposit any phosphorus, which is in keeping with the histological observation that about two days are required for the prehealing cell changes in the epiphyses to be noticeable. This also demonstrates again that diaphyseal deposition is dependent upon mineral concentrations rather than upon vitamin D.

A. T. 10 is practically without effect in curing low-phosphorus rickets in the rat, and not nearly as efficient as calciferol in the low-calcium type. Its action in the latter case has been attributed to an increase in phosphorus excretion (192, 195, 196), but it has been shown to raise the serum phosphorus content and lower that of calcium (193). The small positive effect on calcium retention is probably due to contamination with calciferol; A. T. 10 is not a pure substance (115).

The use of cereal diets for production of experimental rickets has been criticized and discussed above. Purified, complete, noncereal

diets to which phosphorus and calcium can be added as desired are available (76, 205, 206). Experiments with rats on such diets yielded the following results (206). Large amounts of vitamin D given to rats raised on low-phosphorus diets or on normal calcium and phosphorus diets caused kidney calcification which did not occur in the rats on the low-calcium diet. This damage, which was greatest on the low-phosphorus diets, was counteracted by high levels of vitamin A. No increase in the percentage of bone ash could be produced on any diet by large amounts of vitamin D; the levels of calcium and phosphorus and the calcium/phosphorus ratio were the only important factors in this respect, except that small amounts of vitamin D (anti-rachitic doses) and parathyroid hormone produced slight increases in the percentage of bone ash on the low-phosphorus diet. These results can not be explained in any way by the calcium absorption theory of vitamin D action but are quite consistent with the theory that the primary effects of the vitamin, in small doses, are upon metaphyseal deposition of bone and upon reabsorption by the renal tubules; with larger doses the effects are upon the bone shaft. With respect to the action of parathyroid hormone, there is evidence (127) that it also exerts a direct solvent action upon bone. This would be in keeping with the pronounced resemblance between its action and that of A. T. 10.

Pharmacology of calcium.—The rapid injection of calcium salts into human beings results in bradycardia, various degrees of heart block, and an increased excitability of ventricular muscle (207, 208). Successively, in dogs, one finds an inhibition of the heart due to vagus stimulation, a subsequent acceleration due either to sympathetic stimulation or to a direct effect upon the myocardium, and a slowing and arrest due to a direct effect upon the heart (209, 210). It has been mentioned above that calcium salts injected into a calcium-deficient rat will result in a ruptured heart, while ingested calcium will lead to recovery (149). Intravenous calcium gluconate has been found useful in cases of uterine inertia during labor, for it increases the intensity but not the duration of contractions (211).

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BIOCHEMICAL AND NUTRITIONAL STUDIES IN RELATION TO THE TEETH

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The progress of investigation in these fields since they were last reviewed in 1934 has been so extensive that discussions of several topics have had to be omitted, and it has not been possible to mention all papers contributing to the subjects that are reviewed. Those readers who are interested in more complete summaries of the literature of dental caries are referred to the review by Marshall (1) and to the recently compiled abstracts and summaries of conclusions of 237 individual and group investigators of dental decay (2).

CONSTITUTION AND PROPERTIES OF DENTAL TISSUES

Composition.—Considerable variation has been exercised in the selection of materials for study and in the preparation of specimens for analysis. One group of workers has employed extensively a modification (3) of the older Gabriel method by which all organic material is leached out of the calcified tissue by a boiling solution of potassium hydroxide in ethylene glycol. The residue of this extraction is the mineral phase of the material and it has been referred to as the "inorganic tooth substance." Other workers (4, 5, 6), in order to apply the analytical methods to specimens as little altered from their original condition as possible, have employed dry, or fat-extracted dry, specimens. The development (7) of a method for the separation of enamel and dentin [improved by Manly & Hodge (8)] has permitted studies of the composition of these divisions of single teeth.

When available, the values of the standard deviation of the means are indicated in Table I in which are collected the mean results of the more extensive studies of the major constituents of teeth.

A variety of elements, in addition to the major constituents, have been reported in teeth, and it is probable that some of them are of adventitious occurrence. Sodium in a molecular ratio to calcium of 1/30 occurs as a constituent of the mineral phase of enamel and bone (15). About 0.3 per cent potassium has been found recently (16), by arc spectra analysis, as a uniform constituent of enamel; this amount exceeds that which is present in dentin. Spectroscopic evidence (17,

TABLE I
MAJOR CONSTITUENTS OF WHOLE TEETH, ENAMEL, AND DENTIN OF THE HUMAN

Author	Substance Analyzed*	Ca per cent	P per cent	Mg per cent	CO ₂ per cent	Other Substances per cent	Remarks
Crowell, Hodge & Line (3)	W.T.—I.R.†	37.5 ± 9.8	16.3 ± 0.9	0.32 ± 0.25		H ₂ O, 9.2 ± 1.7	Inorganic residue† 81.6 ± 4.7 per cent
LeFevre & Hodge (9)	W.T.—I.R.†	35.2 ± 0.7	16.8 ± 0.3		3.45 ± 0.26	H ₂ O, 8.9 ± 2.2	Inorganic residue† 84.8 ± 2.56 per cent
LeFevre, Bale & Hodge (11)	W.T.—I.R.† Fetal Teeth	34.5 ± 2.3	16.9 ± 0.5	1.1 ± 0.6	3.3 ± 0.1	H ₂ O, 6.81 ± 2.0	Inorganic residue† 80.4 ± 2.0 per cent
French <i>et al.</i> (10) ..	D.†	35.34 ± 0.74	17.09 ± 0.44		3.98 ± 0.38		Sound dentin; 6 sound and 54 carious teeth
French <i>et al.</i> (12) ..	{ E.—I.R.†	37.13 ± 0.99	17.62 ± 0.49		1.98 ± 0.39	I.R.†	Precolumbian specimens
	{ D.—I.R.†	35.70 ± 1.31	16.63 ± 0.94		3.31 ± 0.56	97.8 ± 1.2 I.R.†	
Bird <i>et al.</i> (13)	{ E.—I.R.† Decid.	36.0 ± 2.0	17.8 ± 1.2			80.0 ± 2.3 H ₂ O, 2.8 ± 1.6	Organic† 4.9 ± 3.1 per cent
	{ D.—I.R.† Decid.	33.3 ± 4.6 35.41 ± 0.96 35.64 ± 0.59	16.5 ± 1.2 17.45 ± 0.51 17.21 ± 0.39	0.30 ± 0.05 0.32 ± 0.05	3.00 ± 0.24 3.01 ± 0.14	H ₂ O, 11.1 ± 5.6	
Armstrong & Brekhus (4)	D.¶	26.18 ± 0.34	12.74 ± 0.48	0.83 ± 0.08	3.57 ± 0.10	N ₂ , 3.36 ± 0.14	Sound enamel Carious teeth
Logan (6)	E.—fresh	35.80	16.91	0.25	2.75		Data recalculated to percentage basis
	D.—fresh	26.30	12.70	0.83	3.17		
	E.§—dry	37.07	17.22	0.46	1.95		
Bowes & Murray (5)	D.§—dry	27.79	13.81	0.83	3.17		Pooled material ground from several teeth
Murray & Bowes (14)	{ E.§—dry Decid.	37.07	17.22	0.46	1.95	Cl ₂ , 0.30; ash, 95.3	Pooled material ground from several teeth
	{ D.§—dry	27.79	13.81	0.83	3.17	Cl ₂ , nil; ash, 71.0	

* W.T. = whole teeth; D. = dentin; E. = enamel; Decid. = deciduous; I.R. = inorganic tooth substance (glycol-KOH residue).

+ Ca, P, Mg, CO₂ as per cent of inorganic weight.

† Per cent of dry weight.

18) has been obtained for the occurrence in teeth of silver, sodium, platinum, strontium, barium, chromium, tin, zinc, manganese, titanium, nickel, vanadium, aluminum, silicon, boron, fluorine, and iron. Elements determined by chemical methods are: iron (19) —enamel, 0.0279 per cent, dentin, 0.0087 per cent; zinc (20) —0.02 per cent in whole teeth; fluorine (21) —enamel, 0.0111 ± 0.0018 per cent, dentin, 0.0169 per cent; and chlorine of normal dentin (22), nil. The Van Slyke nitrogen distribution of pooled dentin protein has been described (23). The result for total nitrogen (15.11 per cent) permits the calculation from the results of nitrogen analyses (4) that the average protein content of dentin is 22.11 per cent. The protein content of enamel varies from 0.49 to 1.95 per cent (24). Pincus (25) has objected to the classification of enamel protein with the keratins since it lacks sulphur and exhibits anomalous solubilities.

The high mineral and carbonate contents of mastodon and mammoth tusks (dentin) and the lower content of carbonate in the enamel and dentin of precolumbian teeth (12) in comparison with those of recent specimens have been explained as a result of secondary alterations of the mineral phases; a deposition of calcium phosphate and carbonate in fossil dentin and a partial replacement of the carbonate of the precolumbian enamel and dentin by other anions of soil water are postulated. Logan (6) reported that, unlike human teeth, the carbonate contents of enamel and dentin of dog teeth were nearly equal. Bremer (26), however, in a study of the composition of the separated divisions of the teeth of man, monkey, dog, and swine found considerably more carbonate in the dentin of dog teeth than in the enamel. The low degree of mineralization of dog enamel, found by Bremer (26), may have been a result of imperfect separation of dentin from the enamel since the enamel plate of canine teeth is thinner than that of most other species. Variations in the mineral content of regions of the enamel and dentin of a single human tooth were also noted by Bremer. Murray (27) found rodent teeth to contain more magnesium and phosphorus than dog or human teeth. The incisors and molars of rodents differ in composition chiefly as a result of a greater content of magnesium in the incisors.

The relatively minor variations of composition of whole tooth substance, the small differences in the calcium—phosphorus ratio (2.10 to 2.30) and the very nearly identical x-ray spectrograms (*vide infra*) of whole teeth, enamel, dentin, and bone have been interpreted to indicate a "remarkable constancy" and uniformity of com-

position of the inorganic phase of enamel with that of dentin (3, 9). However, the results of separate analyses of unaltered specimens of enamel and dentin have required a modification, the necessity of which is now generally recognized (10), of the concept of invariable composition of the inorganic phase of the several calcified tissues. Dentin was found (4), in spite of its very much larger protein component, to contain more magnesium and carbonate than enamel. Independent investigations by Logan (6), Bowes & Murray (5), and the later work of Bremer (26) also have demonstrated these differences in the mineral phases of enamel and dentin. Although without significance to the state of combination of the elements, the values of the calculated molecular ratio of tricalcium phosphate to calcium carbonate emphasize the nonidentity of the mineral material of the major divisions of teeth. These ratios, calculated from the mean results of Armstrong & Brekhuis (4), are 4.05 and 2.38 for enamel and dentin respectively. That the mineral matter of enamel contains significantly more calcium and phosphorus than that of dentin, a fact which is not at once apparent from the results of analyses of the protein-containing materials, was made evident from investigations (4, 12, 13) on the composition of the inorganic residues of enamel and dentin from the same teeth. The higher percentage of fluorine in dentin is another significant difference between dentin and enamel (21). In this connection, it is of interest to note that analyses of bone have shown its mineral phase to exhibit a variable composition, and while bone salt is somewhat similar to that of dentin, it differs appreciably from that of enamel (28, 6, 30).

From what has been said above, and because of the variable proportions of enamel, dentin, and cementum in teeth, it is obvious that variations in the composition of either the enamel or the dentin will be obscured when whole teeth are analyzed. Therefore, it is necessary, in any investigation that seeks to discover differences from the normal in the composition of teeth, to analyze separately the enamel and dentin. As a further reason for the same requirement it can be stated that the composition of enamel and dentin and their rates of exchange of elements are influenced, in some cases, by factors that are qualitatively and quantitatively different.

The mechanism that results in the differences in composition of the mineral material of enamel and dentin, and the period in the development or maturation of teeth in which the differences first occur, are not known. It is possible that variations of ionic concentration in

the various tissues undergoing calcification or the action of specific cellular processes (27) may result in the deposition of an inorganic material whose composition is influenced by these factors. It has been suggested (4) that an adjustment toward a uniform composition, and a secondary increase in the magnesium and carbonate contents of dentin, may occur after the formation and eruption of the tooth as a result of the possession by dentin of a mechanism, superior to that of enamel, for interchange between itself and the blood. An increase in the carbonate of bone salt after deposition has been recognized (28) and there is good evidence (29) that the fluorine content of dentin undergoes a secondary enrichment. Even though the results of studies employing radioactive phosphorus also indicate a more rapid rate of exchange on the part of dentin than occurs in the case of enamel, the quantitative importance of secondary alterations of the initially deposited mineral of dentin cannot be ascertained. The lower calcium content of whole inorganic substance of fetal teeth as compared with that of permanent teeth (11) is possibly to be explained, in the light of what has been said above, as a reflection of different proportions of variably mineralized enamel and dentin in the two tooth types.

Investigators of the composition of carious teeth are faced with a dilemma. If the lesion is retained in the specimens examined, variations in composition from that of sound teeth will be found, but they will be in large part a result of the decalcification produced by the carious process and not those that might have contributed to the occurrence of the caries. If, on the other hand, the decayed material is removed, the residual enamel and dentin will differ fundamentally from that of sound teeth only in that they are derived from teeth that exhibited caries. Thus, differences in composition of enamel and dentin which may be associated with susceptibility to decay can be discovered only if the abnormal alterations in composition occur outside of the then existing limits of the lesion. No significant differences in composition with respect to the major inorganic elements of enamel or dentin have been shown to occur outside of the carious lesion (4, 13, 10). Murray & Bowes (14) reported more magnesium in the crown dentin of carious teeth than in that of sound teeth, a finding that had been reported previously for whole teeth. However, these results are at variance with those of Armstrong & Brekhuis (4) and with the recent results of Tefft, French & Hodge (31) which indicated no significant differences in the magnesium content of dentin that could bear a causal relation to caries. Bowes & Murray also

found the dentin of carious teeth, unlike that of sound teeth (22), to contain combined chlorine. It is possible that the presence of chlorine and the increase of magnesium content of dentin, when such increase occurs, may be the result of acquisition of these elements from the oral fluids in contact with the dentin exposed by caries. The higher magnesium content and the presence of chlorine in the roots of pyorrhetic teeth (14) may possibly be accounted for on the same basis. LeFevre & Manly (32) found a significant increase in moisture and organic matter in the sound dentin of carious teeth but could not establish whether the changes from the normal were causal or secondary to the carious process or were a result of the loss, through decay, of the more densely mineralized crown dentin. No changes in composition of the enamel were observed outside of the carious lesion.

Manly & Deakins (33), who made use of micromethods of analysis, found caries of dentin to result in simultaneous decalcification and loss of organic matter, with the former effect predominating in the lesion as a whole. They recognized three zones in a caries of the dentin with different relations of volumes per cent of water, organic material, and inorganic material. Since they found the zone closest to normal dentin to exhibit a proportionally greater loss of organic material than of inorganic substance, some doubt is raised as to the concept that beginning caries is solely the result of decalcification. In this connection, Pincus (34) has claimed to have observed the production of enamel lesions by the action of proteolytic organisms in cultures in which the reaction never became acid.

Submicroscopic structure.—Several investigations (11, 35 to 40) have confirmed the fact, which had previously been recognized, that the powder x-ray diffraction patterns of the mineral of bone, enamel, and dentin are very similar to those of the mineral apatites and that the differences in detail of the patterns are to be accounted for, in some degree, by variations in grain size of the crystallites. Indeed, it has been observed (37, 38, 40, 41) that the diffraction patterns and lattice constants of tooth substance more closely match those of hydroxyapatite than those of other members of the apatite group of minerals. It appears certain, therefore, that the fundamental crystal structure, of at least the predominant part of the mineral phase of calcified tissues, is that of an apatite. The apatites are a series of isomorphous minerals of wide distribution and variable composition. Fluorapatite, which can be obtained pure, has the formula $\text{Ca}_{10}\text{F}_2(\text{PO}_4)_6$. A variety of substitutions for calcium and phosphorus have been recognized and

the replacement of the fluorine of fluorapatite by $(OH)_2$ gives hydroxyapatite. The evidence from crystal structure studies and the good agreement of the reported chemical analyses of whole teeth were interpreted to indicate that the inorganic fraction of teeth has a uniform composition, most probably identical with that of hydroxyapatite. However, the demonstration (*vide supra*) of significant differences in chemical composition of the mineral material of enamel, dentin, and bone brought into question the concept of exact identity of the mineral phases of the calcified tissues and created considerable discussion as to the situation of the carbon in these materials. Gruner & McConnell (42) were able to exclude, for structural reasons, the substitution of carbonate in the apatite lattice at, or between, the positions occupied by fluorine in fluorapatite. This type of substitution formerly was considered possible and the carbonate of calcified tissues was assumed to occupy this position, giving the mixed carbonate-hydroxyapatite frequently referred to, when occurring as an earth mineral, as dahllite. Since the fluorine positions are denied to carbon, Gruner & McConnell (42) suggest that, in order to account for the existence of carbonate apatites, carbon may occupy to a limited degree certain of the calcium and phosphorus positions in the apatite lattice. These substitutions are unusual, but a considerable body of evidence in support of their occurrence has been presented by McConnell (43, 44, 45). In the case of a variety of minerals, the evidence indicates that such substitutions must occur if vacancies in the lattice, so serious as to affect the structure, are to be avoided. This explanation of the structure of carbonate apatites has been criticized on theoretical grounds (37, 46), but has been supported, at least in part, by other workers (46, 47). If the substitution of carbon for phosphorus and for calcium is accepted, the elements of the mineral phase of enamel and dentin (35) and bone (30) can be arranged in the apatite unit cell; and, when complete analyses are available, filling of the theoretical positions and electrostatic neutrality of the statistical distribution can be achieved simultaneously. According to this hypothesis, the mineral material of calcified tissues is a hydroxyapatite in which a variable number of the calcium and phosphorus atoms are replaced by carbon. The general formula (omitting magnesium which can replace calcium in the structure and any sodium and potassium which may be present in the lattice in the calcium positions) can be represented as $(OH)_2Ca_6 \left[[(P,C)O_4]_6(Ca,C)_4 \right]$. The degree of substi-

tution of carbon in the structure is higher in the case of dentin than in that of enamel.

Hodge and co-workers (10, 38, 39) and Bale (40) prefer the view that carbonate apatites do not occur in calcified tissues, and that the sole apatite present in these substances is hydroxyapatite with "occluded, adsorbed, or interstitially crystallized carbonates." Enamel then would differ from dentin only by having a smaller amount of carbonate around, and in association with, the particles of hydroxyapatite. These workers also indicate, especially in the case of dentin, that phosphate ions are adsorbed to minute hydroxyapatite crystals since some specimens of dentin on heating at 900° change their x-ray diffraction patterns (38, 40) to one resembling that exhibited, after ignition, by hydroxyapatite bearing adsorbed phosphate (48). The postulate that some phosphate is adsorbed as phosphate ions reduces the calculated amount of calcium required for the apatite structure, and makes up for the calcium deficit that otherwise would occur as a consequence of the existence of some of the alkali element as carbonate outside of the apatite molecule. Thewlis, Glock & Murray (37) also express the opinion that the carbon of dental tissues and mineral apatites occurs as simple carbonate. They offer in support of their view the fact that, although x-ray diffraction patterns have not shown the presence of calcium carbonate in such materials, they have, by the same token, not demonstrated its absence.

Bale *et al.* (39) found the average size of enamel crystallites to be 0.27 μ ; those of dentin were about one tenth that size. Thewlis has used x-ray diffraction and optical methods in studies of the sub-microscopic structure of enamel and dentin (49, 50). The axes of most of the apatite crystallites of enamel form angles of 5° and 40°, with respect to the direction of the prism, with the first type predominating. Those of the interprismatic substance are mainly of the 40° group, and in dentin (51) the crystallites are arranged in a random manner. From microphotometric tracings of radiographs of tooth sections, Thewlis (51) has shown that there is a steady increase in the degree of calcification of the enamel from the dentino-enamel junction outwards which culminates in some cases in a hypercalcified layer.¹ Dentin exhibits thin layers of low degree of calcification at

¹ Hollander & Saper (52) presented observations which indicate that the evidence for the peripheral hypercalcified layer of enamel is the result of a photographic artifact. Thewlis (51), while admitting that the peripheral "white line" seen on radiographs of enamel is in part photographic in origin, maintains

the dentino-enamel junction and bordering the pulp, but translucent areas associated with caries, or with attrition and secondary dentin, usually are hypercalcified. Warren, Hodge and co-workers (53) have studied intensively the factors influencing the quantitative measurement of x-ray absorption by two slabs and their methods have been employed (55, 56) to determine the regional variations of mineralization of enamel and dentin. Kitchin (57) has reviewed his own work and that of others relating to the study of the structure of enamel and dentin by the use of the polarizing microscope.

Physical properties.—Hodge (58) has developed methods for comparison of the hardness of regions of teeth. The density of most particles of finely pulverized enamel was found (59, 60) to lie between 2.89 and 3.00 while that of dentin and cementum was found, on the average, to be 2.14 and 2.03 respectively. Manly (61) found a strict correlation of refractive index and density of particles of enamel and dentin when the materials were prepared and dried under similar conditions, and Deakins (62) has shown that the ash, organic and water contents of dentin and bone are interrelated. The decreased light scattering coefficient, but normal light absorption coefficient (63), of transparent dentin indicates that it exhibits its properties because of pathological calcification of the tubules. This makes for a structure with a uniform index of refraction, a conclusion that is supported by the fact (64) that the ash content of translucent root tips is hardly altered from that of normal root tips. Volker (65) found that the solubility, at pH 4, of the enamel of deciduous teeth is greater than that of permanent teeth, and that the solubility of the sound enamel of carious teeth is not greatly different from that of sound teeth.

Exchange reactions.—Some of the artificial radioactive elements, particularly the isotope of phosphorus with a half-life of 14.5 days, have been employed to obtain quantitative evidence concerning the much debated question as to the ability of erupted teeth to exhibit, or be influenced by, metabolic processes. Hevesy and co-workers (66, 67) demonstrated an uptake of radiophosphorus by the incisor and molar teeth of rats, and by the teeth of cats and humans. The

that added to this effect is that of an actual increased absorption of x-radiation at the outer edge of enamel. Warren, Hodge, *et al.* (53) have also investigated the peripherally increasing gradient of radiopacity of enamel. Applebaum (54) states that the persistence of the radiopaque white line on the surface of incipient caries indicates that it is not an artifact.

rate of exchange by the teeth was considerably less than that of bone, and the incisor teeth of rats were found to acquire labeled phosphorus both by exchange and by deposition in the actively calcifying root. Manly & Bale (68) obtained similar results, and were able to demonstrate that the phosphorus turnover of calcified tissues occurs in the inorganic portion. Hevesy & Armstrong (69) found a minute fraction of the total active phosphorus, administered parenterally to cats, in the enamel (about 0.005 per cent per gram), and up to ten times this amount in the dentin. Volker & Sognnaes (70) reported, at about the same time, an exchange of phosphorus by enamel, and in a later paper (71) presented evidence that a large fraction of the labeled phosphorus present in the outer layers of enamel is derived from the saliva. They also showed that approximately equal quantities of radioactive phosphorus are acquired by the enamel and dentin of unerupted teeth, contrary to the case of erupted teeth in which the uptake of the tagged element by enamel is about one seventh that of the dentin. Wasserman and co-workers (72), who examined pulpless teeth with and without metal caps over the crown, indicated that only negligible quantities of labeled phosphorus are acquired *in vivo* by enamel from the saliva and showed that about ten per cent of the active phosphorus present in the tooth crown first entered the root by way of the cementum. Recently Barnum & Armstrong (73) found the relative rate of exchange of phosphorus by enamel to be about five per cent of that of dentin. The following percentages of the total quantity of radiophosphorus administered by subcutaneous injection to cats were found after five days in one gram weights of ash: enamel, 0.000546; dentin, 0.00878; femur diaphysis, 0.0133; femur epiphysis, 0.0611; and femur marrow, 0.311. The existence of mechanisms for transfer of phosphate from the saliva through the enamel to the dentin, and from the blood via the dentin to the enamel, were demonstrated. That phosphate migrates outward from enamel and dentin, and at a faster rate from dentin, was shown by the fact that, 116 days after subcutaneous injection of labeled sodium phosphate, the activity of the enamel of the distal half of rat incisors was higher than that of the dentin. Radioactive isotopes of fluorine (74) and sodium (75) have been found to enter the teeth of animals after parenteral administration. The active sodium so acquired by whole teeth was found, in largest fraction, not to be present in the mineral phase. Campbell & Greenberg (76) used radioactive calcium (Ca^{45}) to study the *in vivo* transformations of calcium, and reported that the amounts of

the radioactive isotope acquired by equal weights of bone and whole teeth, on a dry basis, were about equal.

It should be emphasized that the evidence derived from these studies cannot, in itself, be interpreted to indicate an ability of enamel or dentin to undergo significant changes in composition after eruption of the tooth. Most probably the appearance of labeled phosphorus in tooth tissues is the result of an exchange with normal phosphate groups already present in the tissue.² That simple physical processes are the probable bases of the observations is shown by the fact that powdered enamel and dentin can acquire labeled phosphate from solutions *in vitro* (77), and do so in accordance with the Freundlich adsorption isotherm (78). Even though mechanisms for transport of phosphorus into mature dental tissues have been demonstrated, the rates of exchange are such as to indicate that many years would be required to renew the phosphate of one gram of enamel.

FACTORS INFLUENCING THE CONSTITUTION AND INTEGRITY OF TEETH AND INVESTING TISSUES

Schour, in numerous publications, has shown that the continuously growing incisor teeth of rats are a convenient and valuable indicator for the study of the factors concerned with tooth formation. A mid-sagittal section through the incisor records all effects produced in it from the earliest to the final stage. The formation of enamel and dentin of many animals was found to occur in increments of 16 μ width, which in the rat are repeated each twenty-four hours (79), and as four-day phenomena in primates (80). Lesions of the molar teeth of rats can be induced by feeding a diet containing coarse hard particles, and this fact has been the basis of most recent studies of the factors influencing experimental caries. However, several workers have failed to note that many of the lesions so produced actually are fractures (81), and Rosebury (82) has indicated that only occlusal fissure caries of the rat are comparable to those of the human. Even so, the presence of fractured cusps greatly interferes with the scoring of fissure caries (83, 84).

² Structural considerations make it probable that fluorine can substitute for hydroxyl in the apatite structure. Radioactive phosphate acquired by enamel and dentin *in vivo* may result from exchange with normal phosphate groups adsorbed to the apatite crystallites, if such adsorbed phosphate occurs; or the exchange may occur chiefly with the apatite material of the interprismatic substance of enamel and with that of the dentinal tubules.

Fluorine.—The evidence that an unusually high concentration of fluorine in drinking water is the agent causing mottled enamel has been extended. In addition to the foci in other countries, about four hundred areas have been located in the United States in which the condition occurs endemically in varying degrees of severity (85). Dean and co-workers have shown (85, 86) that the continuous use during infancy and childhood of water containing 1 p.p.m. of fluorine will result in very mild mottling of the enamel in about ten per cent of the exposed persons. The incidence and severity of the condition progressively increase when the water contains higher concentrations of fluorine.

Even though mottled enamel may be considered esthetically undesirable, and in spite of the fact that severely affected teeth are structurally inferior (87), the evidence from three diverse sources has indicated that fluorine and resistance to dental caries are in some way associated. Armstrong & Brekhus (88, 89) demonstrated significantly more fluorine in the enamel of sound teeth (0.0111 ± 0.0020 per cent) than in the sound enamel of carious teeth (0.0069 ± 0.0011 per cent), and produced evidence that the difference is not secondary to the caries. Epidemiological studies in Arizona (90), South Dakota, Colorado and Wisconsin (91), Northern India (92), South Africa (93), England (94), and among American Indians (95) have shown a lower incidence of caries in regions of endemic mottled enamel, and thus have substantiated the earlier clinical observations. Other field studies in north-central Illinois (96) and in suburban Chicago (97) have demonstrated an increased immunity to caries in regions in which the fluorine content of the drinking water is high, irrespective of whether or not the teeth are mottled. The inhabitants of Tristan da Cunha are well known to be unusually free of caries. There is clinical evidence (98), supported by fluorine analyses of the enamel and dentin of their teeth (99), that these people display a mild degree of mottled enamel. This occurrence of mottled enamel is unusual in that it does not appear to be water borne. Extradietary fluorine has been reported to reduce the incidence of "corn meal" caries in the rat under the following conditions: (a) When supplied only during the development of the teeth (83, 100). (b) When added in large amounts to the caries-producing diet (84, 101). (c) When given as an aqueous solution (102) or in concentration of 125 p.p.m. (84), 20 p.p.m. (83), and 10 to 100 p.p.m. (103) in the drinking water concurrently with the coarse particle food. (d) When supplied in a concentration of

20 p.p.m. in the drinking water of mature animals for sixty days preceding the caries regimen (83). McClendon & Foster (104) recently have reported a greatly increased susceptibility to decay of the teeth of rats raised on a diet very low in fluorine content.

The mechanism by which fluorine inhibits caries appears to be one of the following, operating alone or in conjunction with the other: (a) An optimal amount of fluorine in enamel confers properties upon it that make it more resistant to caries. (b) The presence of fluoride in the fluids of the oral cavity alters bacterial metabolism and inhibits enzyme action. The former theory is supported by the analyses for fluorine in enamel of caries-resistant teeth and by the continuing protection against caries afforded to the teeth of rats when fluorine is supplied only during tooth development (83, 100) or preceding the feeding of the caries-producing diet (83), as well as by the interpretation placed upon the caries-inhibiting action of fluorine demonstrated with rats deprived of salivary glands (105). As further evidence for (a) may be cited the results of a recent re-examination (106) of the teeth of young residents of Bauxite, Arkansas, who, as children, drank a high-fluorine water. Twelve years after the water supply was changed to a nearly fluorine-free source, the teeth of the persons just mentioned were found to have developed fewer caries than either those of persons who were never exposed to high concentrations of fluorine or those of children born in Bauxite since the water supply was changed. Volker (107, 108) demonstrated that large amounts of fluorine in enamel and dentin reduce their solubility in acid. However, no such effect was produced by the amounts of fluorine present in slightly fluorosed teeth. The second possibility was offered by Miller (101). Dean *et al.* (96) tacitly assumed that this is the principal mechanism since they noted a marked reduction of oral *L. acidophilus* in children continuously exposed to a high-fluoride water. However, the first mechanism cannot be excluded by these studies. In the rat, inhibition of caries produced by high-fluoride drinking water supplied concurrently with a coarse diet has been reported not to be related to the fluorine content of the enamel or dentin (103). The findings of Bibby & Van Kestern (109) with regard to the lessened acid production in cultures of oral bacteria in the presence of as little as 1 p.p.m. of fluorine or of fluorosed enamel and dentin may be interpreted in the light of either, or both, (a) or (b). It has been proposed directly (100) and by implication (96) that fluorine be added to communal water supplies having a low content of this element as a

means of reducing dental caries in the human. The possibilities of hazards to health, not the least of which is the chance of producing severe grades of mottled enamel (87), that would accompany the uncontrolled addition of fluorine to water supplies have been recognized. Therefore, search has been made for a method by which the benefits of fluorine to the teeth can be obtained without the dangers which would be associated with its ingestion. It was suggested by Volker (107) that fluorine might be incorporated in enamel by topical application of fluoride solutions and by Volker *et al.* (110) who demonstrated that powdered enamel and dentin are able to adsorb radioactive fluorine from solutions. The feasibility of this plan is supported by the evidence (29) that the enamel of rat molar teeth apparently is able to acquire fluorine from the oral fluids.

Minerals and vitamins.—Schour (111) has marshalled the evidence that the structure and degree of calcification of fully erupted teeth are not subject to alteration by changes in calcium metabolism, and has indicated that calcium therapy has no justifiable use in the specific treatment of diseases of teeth. Magnesium-deficient diets cause, in a few days, a marked decrease in the absolute amount of this element in the skeleton, but no change in that of rat incisors (112). The inference from this result is that developing teeth are able to acquire magnesium under conditions which do not permit bone to retain it. Acute magnesium deficiency (113) results in a widening of certain zones of the predentin, presumably by increasing the time required for each cycle of calcification. Longer periods of inadequate magnesium intake cause marked disturbances in the character of the enamel (114). Gaunt & Irving (115) have presented other evidence that the teeth may, under certain conditions, be calcified at the expense of bone; and have postulated some fundamental difference in the factors regulating the calcification of the bones and teeth. At low levels of intake of calcium and phosphorus, when the dietary calcium-phosphorus ratio was 4.0, the rat incisor dentin which was formed was nearly normal, even though the calcification of the bones was disturbed markedly. However, when the calcium-phosphorus ratio was 0.5, calcification of the dentin was interfered with at levels of intake of calcium and phosphorus that permitted nearly normal bone formation. The presence in the diet of at least 0.3 per cent of both calcium and phosphorus, for all values of the calcium-phosphorus ratio, was necessary for the formation of normal dentin. Diets very low in calcium and phosphorus do not influence dentin already formed (116).

Irving (117) found that the undercalcified dentin and predentin formed by rats on a rachitogenic diet are little affected by the addition of vitamin D to the diet, and that several days pass before a single vitamin supplement influences the calcification of the forming predentin. Low-calcium, vitamin D-deficient diets produce disturbances in the growth and calcification of enamel and dentin of guinea pig incisors that, unlike the rachitic changes in bones, are not prevented by arrested skeletal growth (118).

Several investigations, too numerous for complete review, have sought additional evidence to show or to disprove the thesis that susceptibility to dental decay is associated with an inadequate intake of vitamin D during, or subsequent to, the period of tooth formation. Extra vitamin D, supplied in various ways concurrently with a coarse particle diet, has been reported (119) to reduce the incidence of fissure caries in the rat; a similar effect, independent of that of vitamin D, was noted to result from the incorporation of corn oil and other fats in the diet. Mellanby and co-workers (120, 121) have restated and extended their evidence that dietary deficiencies, particularly a deficiency of vitamin D, are present almost universally among the factors which contribute to the susceptibility of teeth to decay. They hold that irregularities in surface texture of human teeth are one manifestation of abnormal tooth structure produced by an inadequate supply of vitamin D during tooth formation, since similar effects have been produced in the teeth of dogs by rachitogenic diets; furthermore, the susceptibility of such teeth to decay is proportional to the degree of surface abnormality (122). The term "hypoplasia" is used to describe the abnormal tooth surface, but Lady Mellanby emphasizes that she refers to conditions much less severe than those usually connoted by this word. Observations of the effect of various forms of vitamin D supplements to the diets of children in institutions have been interpreted to indicate that a high vitamin D content of the food diminishes the incidence of caries if the vitamin is given during tooth development; and that the onset and spread of caries can be delayed even if the vitamin is given after the teeth are formed (123). Objections to the evidence and conclusions of Mellanby *et al.* have been offered by Weaver (124). The findings of Taylor & Day (125), with reference to certain children in India who displayed the stigmata of severe rickets but very few caries, also indicate that lack of vitamin D cannot be the sole cause of caries.

That the primary effect of vitamin A deficiency on developing

teeth is on epithelial tissues has been confirmed by Schour and co-workers (126) who described, as a result of vitamin A deficiency, an acceleration of the rate of apposition of the enamel-covered dentin but an opposite effect on cementum-covered dentin. Working with rats, Mellanby (127) found that prolonged vitamin A deficiency of the mothers produced, in the offspring, marked disturbances in the structure and calcification of the incisor enamel, and irregularities in the development of the dentin that were regarded as secondary to the enamel hypoplasia. King (128) produced in dogs, by feeding vitamin A-deficient diets, delay in eruption, irregularities in the alignment of the teeth, and malformation of the alveolar bone. These effects were not entirely duplicated by section of the inferior alveolar nerve.

The well-known effects of avitaminosis-C on developing dentin and the pulp have been reinvestigated (129, 130). Boyle (131) has shown that defective enamel formation in the incisor teeth of scorbutic guinea pigs, which Fish & Harris (130) claimed to be a primary effect of severe ascorbic acid deficiency, actually is secondary to retarded dentin deposition and to failure of collagen fibers and bone matrix to form in the periodontal tissues. The rate of dentin formation in the incisor teeth of guinea pigs was found (132) to be proportional to the intake of ascorbic acid up to a daily dose of 5 mg. Those animals receiving less than 0.75 mg. per day deposited irregularly formed dentin. The pathological changes produced in the jaws of guinea pigs by diets deficient in ascorbic acid have been reported (133) to be very similar to that type of pyorrhea in the human characterized by diffuse alveolar bone atrophy. No clear evidence has been presented that vitamin C deficiency is associated with caries in the human, but King *et al.* (134) have shown that a toxic state (diphtheria toxin) increases the ascorbic acid requirement for normal dentin formation.

Endocrine factors.—Hypophysectomy in the rat markedly decreases the rate of eruption of the incisors but interferes to a much less degree with the formation of enamel and dentin; the latter, incidentally, becomes overcalcified (135). Similar effects, modified by their limited growth, were seen in developing molars (136). Parathyroidectomy produced (137) a hypercalcification of the dentin formed during the first twenty days following operation, but the dentin subsequently formed was calcified imperfectly. Longer periods of survival resulted in changes in the structure of the enamel and dentin that were aggravated by periods of fasting or pregnancy. The administration of parathormone or massive doses of calciferol to para-

thyroidectomized rats produced (138), in the incisors, primary hypocalcification of the dentin, followed by hypercalcification of the dentin subsequently deposited. It has been concluded, therefore, that massive doses of calciferol do not require the presence of the parathyroids to exert their effects. Bilateral adrenalectomy causes (139), in the rat, premature calcification of the predentin and overcalcification of the postoperative dentin. The somewhat similar results noted after removal of the pituitary may be due to a secondary adrenocortical insufficiency. The dental changes in the rat produced by adrenalectomy were found (140) not to be associated in any manner with changes in the storage or metabolism of ascorbic acid. The administration of thyroxine, but not pituitary thyrotropic hormone, to rats induced precocious eruption of the incisors (141). Ziskin & Applebaum (142) found thyrotropic hormone to accelerate, and thyroid ablation to retard, the rate of dentin apposition in the permanent teeth of young rhesus monkeys. Thyroidectomy also resulted in imperfectly calcified dentin. Administration of anterior-pituitary and castrate-urine-gonadotropic substances to normal monkeys, and estrogenic hormones to ovariectomized animals, produced proliferation and keratinization of the mucosa of the gums (143). Pregnancy-urine-gonadotropic substance produced degenerative and inflammatory changes in these tissues of spayed monkeys. Somewhat similar effects were seen in the gingival tissues of women treated with pregnancy-urine extract and with estradiol benzoate (144).

Local factors.—Brawley (145), using colorimetric methods, found the pH of normal resting saliva to range from 5.6 to 7.6 (median value, 6.84; average value, 6.75). A slight diurnal variation of the results, associated in time with meals, was observed. Becks & Wainwright have published extensive critiques of the literature with reference to the calcium (146) and phosphorus (147) contents of saliva, and have described improved methods of analysis for these elements in saliva. Karshan (148) examined stimulated and unstimulated saliva from persons free of active caries, and from persons with active tooth decay. He reported statistically higher mean values in the caries-free group with respect to carbon dioxide combining power, total calcium and phosphorus contents, and percentage of calcium removed from the saliva by agitation with tricalcium phosphate. White & Bunting (149) found resting saliva to contain higher concentrations of calcium and phosphorus than stimulated saliva, but no relationship between the composition of saliva and caries susceptibility was noted. Recently,

Becks *et al.* (150) reported the following average results from the analyses of the resting salivas of 90 caries-free individuals: rate of flow, 19 cc. per hour; calcium, 6.48 mg. per 100 cc. (1.14 mg. per hour); and phosphorus, 15.33 mg. per 100 cc. (2.76 mg. per hour). No significant variations from these values were found when the salivas of 118 persons with active caries were examined. Since variations of rate of flow, character, and composition of saliva occur under diverse conditions of stimuli, it has been difficult to define "normal saliva." This difficulty and the fact that caries usually are insidious in onset and slow in progress make it evident that subtle changes in composition of saliva, or those changes in saliva of transient occurrence, which might be associated with susceptibility of teeth to decay, will be almost impossible to discover.

Stephan (151), using an antimony electrode in the mouth, found the average pH of mucin plaques and open cavities to be 6.2 and 5.5, respectively. In both cases significantly lower values were found after rinsing the mouth with a glucose solution, the maximum effect being noted in the first 30 minutes. Grossman (152) found the pH of aqueous suspensions of sound dentin to be 8.16 ± 0.22 and that of carious dentin to be 6.51 ± 0.36 .

Lactate (153) and, after the ingestion of sugar, lactate and pyruvate (154) have been demonstrated to be present in carious tooth material by quantitative methods. An increase in the lactic acid content of saliva following a carbohydrate meal has been reported (155). Fosdick and co-workers (154, 156), using controlled conditions of fermentation of glucose by salivary organisms, have been able to identify several of the intermediate products of carbohydrate degradation that are known to be formed in the cycles of utilization of glucose by yeast and by muscle. It was suggested, therefore, that acids other than lactic, for example pyruvic and phosphoglyceric, may be important agents in the decalcification of dental tissues. The enzyme systems of oral yeasts and *L. acidophilus* were found to be complementary. The initial rate of acid formation from carbohydrate in carious lesions was found (154) to be rapid, but the process was of limited duration. This last finding would indicate that oral hygiene procedures, or measures to control acid production, would need to be applied immediately after eating sugar; further, that only by repeated ingestion, as Stephan (151) had previously intimated, can carbohydrate influence the progress of caries.

The conclusions of the Michigan workers in regard to the diag-

nostic relation of salivary lactobacillus counts to caries activity, and of the influence of carbohydrate in promoting the oral growth of these organisms, have been restated and reviewed (157). King & Croll (158) did not corroborate either of the above conclusions, and Boyd and co-workers (159) have once more been unable to confirm an etiological relationship between *L. acidophilus* and dental caries. Nevertheless, a high degree of correlation of salivary *L. acidophilus* counts with occurrence of caries has been noted in two of the studies (96, 97), which have established the relationship of resistance to caries and the fluorine content of communal water supplies. Recently, Arnold & McClure (160) reported a close correlation between the caries activity of a group of children, whose teeth were presumably not influenced by unusual amounts of fluorine, and the count of *L. acidophilus* organisms in the mouth. No relationships of caries activity or bacterial counts with variations of a number of salivary properties and constituents were noted. The arrest of dentinal caries of children by dietary control again has been reported by Boyd (161), and it was stated that in this study about half of the calories of the diabetic diets employed were supplied as carbohydrate.

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THE CHEMISTRY OF VISUAL SUBSTANCES

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VISUAL PURPLE AND VISION

Historical.—After the discovery of photography early in the 19th century Moser (1) suggested that the retinal basis of vision is probably a photochemical process. Though this notion was generally accepted, its demonstration was not made until thirty-five years later when Boll (2) found that the pinkish color of a freshly removed frog's retina gradually bleaches in the light. A year later Kühne (3) actually extracted a reddish substance from the retina with a solution of bile salts and showed that it bleached in light. This photosensitive material was called rhodopsin or visual purple, and its existence raised high hopes for understanding the chemical basis of the visual process.

Kühne did a prodigious amount of experimentation with visual purple; indeed much of subsequent work by others merely carried out with precision those experiments which he made with rough methods, and the meaning of which he appreciated fully. In spite of this, interest in visual purple declined rapidly. One reason was that Kühne's industry and acumen had pushed the investigation to the margin of contemporary chemical knowledge, and therefore it had to await the general growth of chemistry. Another reason was that visual purple was found only in the rods and not in the cones. Since the region of sharpest vision contains only cones and no rods, visual purple could not be considered essential for good vision. Moreover, frogs whose visual purple had been completely bleached by sunlight were still capable of the most accurate vision.

In the subsequent forty years some work was still done with visual purple. Koenig (4) measured the absorption spectrum of human visual purple, while Koettgen & Abelsdorff (5) investigated different animals, and found two distinct types of the photosensitive material. Also Trendelenburg (6) and Garten (7) studied its bleaching in different parts of the spectrum. In the main, however, work in vision turned to channels such as color perception in which physics was more necessary than chemistry.

Chemistry again became of interest in vision twenty-odd years

ago when Lasareff (8), Pütter (9) and Hecht (10) made efforts to describe the quantitative data of visual function in terms of photochemical and chemical knowledge. These schemata assume that at least three chemical steps must take place early in visual stimulation: (a) a primary photochemical process in which a substance absorbs light and is changed by it; (b) a primary "dark" reaction which replaces this photosensitive material either from its products or from other precursors; and (c) a secondary "dark" reaction by which the primary photoproducts, either by themselves or with the help of other substances, initiate a nerve impulse. It is astonishing how adequate a quantitative description of many visual functions may be given by the properties of a reversible system comprising even only the primary light and dark reactions. This is especially true of visual properties measured at the photostationary state corresponding to adaptation of the eye to various light intensities.

The literature of this period has been reviewed elsewhere (11, 12), and I mention it here only because it formed the basis for the new interest and importance of visual purple. This came about in two ways. The first was through the modern growth of the Duplicity Theory (13, 14, 15), in terms of which the vertebrate retina is considered a double sense organ: the rods are concerned with colorless vision at low light intensities while the cones are concerned with color vision at high light intensities. The rods thus take on independent sensory status, and visual purple with them. In the second place, all these researches show that in spite of the obvious differences between rod vision and cone vision their behavior in such functions as intensity discrimination, visual acuity, flicker, instantaneous threshold, and dark adaptation is remarkably alike quantitatively and may be described theoretically by very similar equations. Therefore if visual purple is really the photosensitive material of the rods, then its chemical behavior in solution may not only be applied to rod vision, but may serve in addition as a model for the unknown substances in the cones whose kinetics and dynamics cannot be very different from it.

Absorption spectrum and luminosity.—Visual purple has been found in the whole vertebrate series from Petromyzon to man. From the excellent summary by Garten (16) it appears that visual purple is always plentiful in predominantly rod retinas, and occurs only sparsely or not at all in predominantly cone retinas. In mixed retinas like that of the human eye it is observed only in the regions outside of the fovea; that is, in locations which contain rods. Microscopic

observation shows it to be located only in the outer segments of the rods. Since visual purple is morphologically associated only with the retinal rods, its function must be looked for only in connection with rod vision.

Kühne had noticed the rough coincidence between the spectral absorption of visual purple and the brightness distribution in the spectrum for the human eye. However, it was not until Koenig measured the absorption spectrum of human visual purple with his newly invented spectrophotometer and also first determined the spectral sensibility distribution of the eye at low illuminations that the similarity between the two became apparent. If the two were exactly alike, it would mean that in order to produce a given visual effect in the spectrum the same amount of energy must be absorbed by visual purple regardless of the wave length. Moreover, Trendelenburg found that the effectiveness of the spectrum in bleaching visual purple solutions runs fairly parallel with its absorption spectrum and with the sensibility distribution of the eye in the spectrum at low illuminations. This shows that a given visual effect represents not only a given absorption of energy but a given photochemical effect.

At best, these were first order measurements. A precise determination of the sensibility distribution in the spectrum at low intensities was made by Hecht & Williams (17) with forty individuals, and has been confirmed later by Sloan (18) and more recently by Weaver (19). This represents the reciprocal of the energy at any wave length required to produce a constant effect in rod vision, and is called the scotopic luminosity curve. Its maximum is at 511 $m\mu$, and it falls off smoothly and nearly symmetrically toward both ends of the spectrum. Its shape is almost exactly that of the commonly used, symmetrical absorption spectrum of visual purple as given by Koettgen & Abelsdorff (5), by Chase & Haig (20) and by Lythgoe (21). However, the absorption maximum of visual purple lies very near 500 $m\mu$, and for years this difference of 10 $m\mu$ between absorption maximum and spectrum visibility maximum lacked an adequate explanation.

The discrepancy becomes worse when it is realized that the absorption spectrum of visual purple as usually given is not its real absorption spectrum. The classical absorption spectrum is the difference between the spectrum of the unbleached solution and of the completely bleached solution, and was used because of the impure and uncertain nature of the retinal extracts. However, the most purified extracts of visual purple prepared by Lythgoe (21), Saito (22), Chase

& Haig (20), Wald (23), and Krause & Sidwell (24) yield an unsymmetrical absorption spectrum. In Figure 1 are collected the absorption

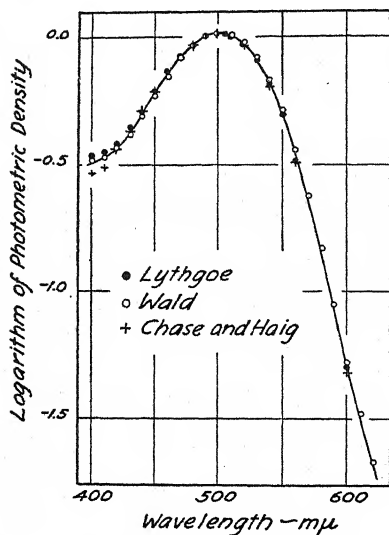


FIG. 1.—Absorption spectrum of visual purple. The points are independent measurements on separate preparations by Lythgoe in London, by Wald at Harvard, and by Chase & Haig at Columbia. Except in the extreme violet, where measurements are difficult and uncertain, the agreement is practically perfect.

data of frog's visual purple from three sources; they show how adequately characterized this substance has now become. The high absorption in the blue is not due to impurities, but to visual purple itself, because during ultracentrifugation its rate of sedimentation as measured in blue light is identical with the rate in green light (25). Moreover, Schneider, Goodeve & Lythgoe (26) have found that the bleaching rate of visual purple in the spectrum runs parallel with the absorption spectrum of purified visual purple rather than with the difference between the spectra of bleached and unbleached solution.

Fortunately, these differences in symmetry and position between the scotopic luminosity curve and the absorption spectrum of visual purple are now completely understood in terms of two corrections which must be applied to the luminosity data. The first involves the realization that photochemical action is a quantum effect. Dartnall & Goodeve (27) have recomputed the luminosity curve in terms of the relative number of quanta instead of the relative energy required for a given visual sensation, and find that its maximum shifts distinctly toward the blue.

The second correction recognizes that luminosity data are measured at the cornea, whereas visual purple is in the farthestmost interior

of the eye. Measurements by Roggenbau & Wetthauer (28) and by Ludvig & McCarthy (29) show that the ocular media have relatively high absorptions in blue and violet light, high enough so that when ocular absorption is applied to the quantum luminosity curve, it corresponds with precision to the absorption spectrum of purified unbleached visual purple (23, 30). As a result it is now established beyond doubt that visual purple is the photosensitive material of rod vision.

Concentration and sensitivity.—When the eye is exposed to bright light for some time its light sensitivity decreases. Under such conditions, the retina loses its color and its visual purple concentration is decreased. On return to darkness the eye gradually recovers its sensitivity; similarly the color of the retina returns and its visual purple concentration increases.

In the human eye complete recovery of sensitivity takes about an hour, though the process is practically finished in thirty minutes. In the eyes of the intact frog, regeneration of visual purple takes several hours at room temperatures, while in the rabbit it is complete in about forty minutes (31). Since the rate of regeneration is influenced by temperature (32, 33), these two observations lead to about the same rate of regeneration at body temperatures. In fact, the regeneration rate of visual purple is not very different for different animals at the same temperature (16). An exception is the white rat in which Tansley (34) found complete regeneration only in about four hours. In general, however, human visual dark adaptation runs roughly parallel with the accumulation of visual purple in the dark adapting animal retina.

Efforts to study this parallelism experimentally have not been successful. Using the electrical response of the eye to a standard light as a measure of its photosensitivity, Granit, Holmberg & Zewi (35) found that frog eyes exposed to light long enough to reduce the electrical response to a quarter of its dark value show no difference in visual purple concentration as compared to dark adapted animals. This led them to the unlikely suggestion that only a thin layer of visual purple on the surface of the rod is active photochemically, the main bulk of the material serving as reservoir. Later they (36) reported that when the electrical response has fallen to between 10 and 40 per cent of the dark value, the concentration of visual purple has also dropped to about 10 per cent. However, the subsequent course followed in the dark by the electrical response is apparently quite dif-

ferent from that of visual purple concentration; the former shows a long delay before it begins to rise, while the latter begins to increase at once.

These somewhat contradictory findings of retinal potential behavior are probably correct, though they are quite at variance with the observations of Riggs (37) who finds that during dark adaptation of the frog the retinal potential begins to increase immediately after light adaptation. However, a great deal still remains to be learned about the relation of retinal potentials to vision before they can be used to correlate changes in visual sensitivity with alterations in visual purple concentration. In fact, even the sensitivity data of human dark adaptation, though very precise, are still without adequate theoretical treatment in terms of visual purple concentration changes (38). It has therefore seemed desirable to pursue direct chemical studies of visual purple itself.

CHEMICAL NATURE OF VISUAL PURPLE

Solubility.—Visual purple was first extracted from retinas by solutions of bile salts (3). In recent years a number of additional substances have been found to be effective as extractives. They are digitonin and saponin (34), panaxtoxin, saponin, sodium oleate, and sodium salicylate (39), and sodium desoxycholate (20). Of these, the oleate and salicylate are the poorest, and saponin is not much better; the extracts are dilute and often cloudy. The best extractive is still sodium glycocholate. However, because its preparation in colorless form is of classic difficulty in organic chemistry, most modern workers use digitonin or sodium desoxycholate which may be bought in colorless form. Sodium desoxycholate has the property that extracts made with it, and bleached, do not regenerate in the dark (20, 40).

Kühne originally believed that the bile salts merely served to break up the rods. The cytolytic power of saponin, digitonin, and the other extractives would seem to bear this out. However, he found that during dialysis of the extract the colored substance is precipitated. With one exception (41) this has been confirmed not only with glycocholate extracts but with the other extractives as well (20, 42). Evidently these substances are necessary to maintain visual purple in solution and this probably depends on their mildly detergent properties. Increasing the concentration of digitonin does not increase the concentration of visual purple in the extract, but it does markedly increase the stability (20).

Extracts prepared from whole retinas are most frequently opalescent and undoubtedly contain many other substances. In addition they do not keep well even at low temperatures. The purity, clarity, and stability of the solution is tremendously increased by Kühne's device of a preliminary hardening of the retinas in 4 per cent alum solution for several hours. Evidently the alum renders much of the retina insoluble, and the subsequent extracts are reddish crystalline-clear solutions which in our experience may remain clear for weeks at room temperature, and even for years at 0° C. without extensive deterioration. Judged by their absorption spectra (20), such solutions are free of blue-absorbing impurities and show only one sedimenting boundary in the ultracentrifuge (25).

The purity of the solution may also be enhanced by controlling the pH and temperature of the extractive. The purity is greater, the lower the pH down to 5.5, and the higher the temperature up to 40° C. (20).

Occasionally, weakly colored retinal extracts are secured with water alone. In Kühne's experience, as well as in my own, these are fine suspensions of rod fragments, because the color may be filtered off or settled by centrifuging. Chase & Haig have reported a water extract which did not become colorless on ordinary centrifugation, and Dartnall, Goodeve & Lythgoe (43) report a water solution prepared from retinas that had been repeatedly frozen and thawed. These solutions, however, were opalescent; and Chase & Haig's solution deteriorated rapidly even though its origin was alum-treated retinas. In fact, its absorption spectrum was high in the blue, much like that of the retina itself or of poor extracts, rather than like that of extracts from alum-treated retinas, and this speaks for its having probably been a very fine suspension of rod fragments.

Preparation.—The purest and most stable solutions may be prepared in several ways. The simplest is from whole retinas. The animals are kept in the dark, usually over night. All subsequent procedures are made by the light from a dim ruby lamp or in total darkness. The animals are decapitated and the eyes removed. Each eye is cut through just back of the iris, and the lens and vitreous are removed. With frog's eyes the retina may be lifted out at once and put into water or salt solution. The accumulated retinas are centrifuged and the supernatant liquid discarded. They are then placed in 4 per cent alum for several hours, after which they are washed first with buffer solutions and then with water until all the alum is removed.

Mammalian eyes, after removal of lens and vitreous, are best placed in alum at once, since the hardening process makes it easy to remove the very delicate retinas. These are then treated like the frog retinas. After they have been washed, they are put into 2 to 4 per cent sodium glycocholate, or digitonin at a slightly acid pH at room temperature or slightly above in a ratio of about twenty frog retinas to 1 cc. of solution, and left with occasional stirring for about two hours. The mixture is then best centrifuged in the cold, and the clear red supernatant liquid removed. At this point buffer should be added to bring the pH to approximately 7.

With frog and toad retinas one may take advantage of the ease with which the terminal segments of the rods come off. Lythgoe (21) merely shakes the retinas violently in water to liberate the segments, while Saito (22) recommends a 40 per cent sucrose solution which after centrifugation leaves the segments in a layer on top of the solution. Lythgoe treats the sedimented terminal segments with petroleum ether and then dries them before extracting with digitonin; this removes some yellow impurities. Judged by their absorption spectra especially in the blue, solutions prepared in this way are only slightly, if at all, purer than those prepared from alum-treated whole retinas. In Saito's case they are not as pure.

Chemical and thermal sensitivity.—Most of the common laboratory reagents rapidly destroy the color of visual purple. This fact accounts for the delay in its purification and in the acquisition of chemical information about it. Substances like ether, acetone, ethyl alcohol, heavy metal chlorides, formaldehyde, strong acids and alkalis, and chloroform bleach it at once. On the other hand, dilute ammonia, sodium carbonate, sodium chloride, urea, and even alum and hydrogen peroxide leave visual purple untouched. It is apparently insensitive to trypsin (3).

Heat destroys the color; judging by Kühne's measurements, the temperature coefficient (Q_{10}) of the thermal destruction is near 7. According to Wald (44) Kühne's data for the velocity of heat destruction in the fresh retinas fit the Arrhenius equation with a thermal increment of 75,000. New measurements by Lythgoe & Quilliam (45) show that the thermal destruction of visual purple solutions also follows the Arrhenius equation, but yields a thermal increment of only 44,000 calories. The rate of thermal bleaching is best expressed as a first order reaction. The thermal change is retarded by an increase in salt concentration, and the rate at both ends of the pH range varies

approximately as the square of the hydrogen ion and of the hydroxyl ion concentration.

Visual purple as protein.—It is apparent that visual purple behaves to heat and chemicals as do proteins (44, 46). Moreover, even the purest solutions of visual purple contain mostly protein material (42, 47). Such solutions show an ultraviolet absorption curve which is characteristic of proteins, and which is continuous with its absorption spectrum in the visible (24). Visual purple can be salted out of solution with saturated sodium sulfate and magnesium sulfate, neither of which alters the substance, since it may be redissolved in water, but with apparently no gain in purity (20). The dialyzed precipitate, if dried, becomes insoluble, though it may preserve its color and sensitivity to light for long periods.

Visual purple is amphoteric and shows an isoelectric point (48, 49). Solutions dialyzed to remove most of the salt, when measured in an Abramson apparatus, yield an isoelectric point at pH 4.47 for the unbleached and 4.57 for the bleached condition. The same value is reported by Wald (23) for grass and bull frog visual purple measured by the moving boundary method.

The protein nature of visual purple is further attested by its slow diffusion through porous membranes. Its diffusion coefficient is 0.0148 sq. cm. per day, which in terms of the usual computations yields a molecular weight of 810,000 (50). The molecular weights of proteins calculated from diffusion data are often too high by a factor of two or three compared to those derived from osmotic pressure or sedimentation data. This turns out to be the case with visual purple. Measured in the ultracentrifuge (25), it shows a sedimentation constant of 11.1×10^{-18} cm. per dyne per sec., which corresponds to a molecular weight of about 270,000. However, this result is possibly complicated by the behavior of the digitonin in the solution, since this substance seems to form micelles whose particle weight may be as high as 75,000 or more (51).

All this evidence furnishes powerful proof that visual purple is really a protein. The kind of protein it is may be judged by Kühne's inability to separate it from the neurokeratin of the rods. Its insolubility in the usual protein solvents like water, dilute sodium chloride solution, and even alcohol, confirms its similarity to these insoluble proteins.

Visual purple as conjugated protein.—Visual purple, however, is not a simple protein; its color alone suggests its conjugated nature,

since most simple proteins are colorless, and all common colored proteins are conjugated. Wald (23, 44) has shown that visual purple is a carotenoid protein. The experiments are sufficiently important and sufficiently simple to be reported in detail.

Dark-adapted retinas when extracted with petroleum ether yield exceedingly small amounts of vitamin A. If, after such treatment, the retinas are extracted with chloroform, they lose their color and yield to the chloroform a greenish yellow pigment which exhibits carotenoid properties and shows absorption bands at 280 and 310 m μ . With antimony trichloride this material reacts to give a blue color corresponding to a single absorption band at 664 m μ . This location of the band is unique and corresponds to no previously known carotenoid—a fact confirmed by Minoshima, Hosoya & Ishii (52). Wald has called the substance retinene. Bruner, Baroni & Kleinau's (53) identification of this carotenoid as β -carotene has not been confirmed by Wald (44, 54, 55) or by Krause (47). Retinene, after extraction with chloroform, is freely soluble in petrol ether and in carbon disulfide; however, neither of these solvents can extract it from the dark-adapted retina. Chloroform, in bleaching the visual purple, evidently renders the retinene available for solution in petrol ether.

The change in availability of retinene is also accomplished by light. Dark-adapted retinas when exposed to light become orange yellow. In this condition they yield their full content of retinene to petrol ether and to carbon disulfide. Evidently both light and chloroform, when they bleach visual purple, make the retinene easily extractable by such gentle solvents as petrol ether.

Retinene, though previously unknown, is related to vitamin A. Wald has found that after bleached retinas have been allowed to fade at room temperatures, the retinene which they contain steadily decreases in concentration and is replaced by vitamin A which increases in amount. In wholly bleached and faded retinas there is no retinene, but considerable amounts of vitamin A, about 1 to 4 μ g. per retina depending on the species. Measurements show a regular relation between the two processes, and suggest that the conversion of retinene to vitamin A in the isolated retina is stoichiometric. It is a thermal reaction as shown by the decrease in its speed at low temperatures. This relation is of great importance for the visual process because vitamin A is necessary for the normal formation of visual purple. It is thus both a precursor and a product of the visual cycle.

These relations between visual purple, retinene, and vitamin A

have been established for the isolated retina. In the intact animal the simple relationships here described are complicated by the presence of the pigment layer in contact with the retina. This layer contains about 1 μg . xanthophyll, and about 4 μg . vitamin A (44, 54, 55) per eye. Moreover, the blood supply which also contains vitamin A enters into the system. Thus frogs which have been in bright light yield retinas which are colorless, but which contain no retinene, and only about 0.2 μg . of vitamin A per retina.

Helmholtz (56) and Setchenow (57) found that the light-adapted retina fluoresces in the ultraviolet, and later Himstedt & Nagel (cf. 16) showed that the bleached products of visual purple fluoresce similarly. This green fluorescence is undoubtedly due to vitamin A, and has recently been demonstrated again microscopically by Jancsó & Jancsó (58) in the retinas of light-adapted albino rats, but not in those of dark-adapted animals.

Visual purple solutions have not been sufficiently investigated. Brunner, Baroni & Kleinau studied the visual purple prepared from bile salts solutions of cattle and pig retinas by dialysis and subsequent drying in vacuo. Extracts with petrol ether gave no carotenoid reactions, thus confirming Haurowitz (59). However, if the solid visual purple is first treated with a few drops of alcohol, it is bleached at once and a petrol ether extract of it shows carotenoid properties. Brunner, Baroni & Kleinau speak of this as carotene, meaning probably β -carotene which they report from bleached retinas, as do Euler & Adler (60); however, in view of the work of Wald (44) as well as of Krause (47, 61) and Minoshima, Hosoya & Ishii (52), this identification is most probably incorrect.

Krause (47) finds that the solid material from a solution of visual purple from cattle contains three-fourths protein and one-fourth "lipin" of undefined nature. An unbleached visual purple solution yields no carotenoid to petrol ether; after bleaching by light to golden yellow it gives up material to petrol ether which reacts with antimony trichloride. However, if the bleaching is continued, the solution becomes paler and the petrol ether extract gives no reaction to antimony trichloride. Thus according to Krause, bleaching the solution by light first liberates a carotenoid, but further exposure to light changes the material so that it loses its carotenoid properties.

These observations are only partially confirmed by Wald (23). Visual purple solutions may be precipitated in the cold with acetone without bleaching. Neither the precipitate nor the supernatant solu-

tion yields any carotenoid to petrol ether. After bleaching of solutions by light, the precipitate with cold acetone contains not only the protein, but most of the yellow color. The retinene must therefore still be mainly attached to the protein, since carotenoids are not easily adsorbed in the presence of high concentrations of acetone. To liberate the retinene, the petrol ether must contain 1 to 2 per cent alcohol. This probably explains why Lythgoe (21) was unable to extract carotenoids from partially and completely bleached solutions by petrol ether alone.

Protein and prosthetic group.—As the result of all these observations it is safe to suppose that visual purple is a conjugated carotenoid protein. Carotenoid proteins have been well known for decades (62), and many new ones have been described and studied in recent years (63), the most recent one being ovooverdin, the green pigment of lobster eggs which has some similarities to visual purple (64). Visual purple, however, seems to be the only one of the group which is sensitive to light. This light sensitivity is undoubtedly related to the particular prosthetic or chromophore group in the molecule. In the case of visual purple the chromophore group is probably retinene or some immediate precursor, since any action by light or other agents which bleach visual purple loosens the bond between the carotenoid and the protein and liberates retinene.

The number of prosthetic groups per molecule of protein has been determined for a variety of chromoproteins, and from these measurements Broda, Goodeve & Lythgoe (42) have computed the carrier weight of chromoprotein for a single prosthetic group. Nearly all of these are respiratory proteins. For about a third of them the carrier weight lies near 17,000, or the weight of a Svedberg unit; for the rest, the carrier weight ranges from 25,000 upward. Making certain entirely reasonable assumptions, Dartnall, Goodeve & Lythgoe (65) have estimated the molecular absorption coefficient of visual purple as about 2.3×10^4 . Then knowing the photometric density of a solution of visual purple and determining the amount of protein nitrogen in it, Broda, Goodeve & Lythgoe have found the carrier weight of visual purple to be about 27,600; if certain corrections for impurities are made, this value comes to about 26,500. Remembering Hecht & Pickels' determination of the molecular weight as 270,000, this gives about 10 prosthetic groups per molecule of visual purple.

In terms of these findings and of the number of rods involved, Broda, Goodeve & Lythgoe have estimated that the terminal segment

of each rod in the frog's retina contains about 10^8 molecules of visual purple. These would occupy about 10^{-10} cc. and since the volume of a terminal rod segment is about 10^{-9} cc., this means that visual purple occupies about 10 per cent of the volume of the outer limb of the rod.

The concentration of visual purple in the human retina is much less than in the frog's, and the terminal segments of the human rods are also smaller than the frog's. However, a factor of 10 easily takes care of this and it can safely be assumed that each human rod contains at least 10^7 molecules of visual purple. Nevertheless, we can see a flash of light when only one molecule in each of 5 to 7 rods is changed (66). Apparently the rest of the rod must be concerned with amplifying this photochemical effect to sufficient magnitude so that it will initiate a nerve impulse.

VISUAL PURPLE BLEACHING

Photochemical process.—The first quantitative determinations of the bleaching of rhodopsin by light were by Trendelenburg (6), who followed spectrophotometrically the relative action of different parts of the spectrum. Interest at that time was in the relation of visual purple to vision, and he was able to show that the bleaching rates in the spectrum are fairly proportional to the relative effectiveness of the same spectrum on low-intensity rod vision. The measurements are excellent, and some of them, when recomputed by Schneider, Goodeve & Lythgoe (26), show good agreement with their own data on the quantum efficiency of rhodopsin bleaching.

The kinetics of the photochemical bleaching are simple. The disappearance of visual purple in solutions exposed to light follows the course of a first order reaction without any induction period (67). This is understandable if the speed depends on the amount of light absorbed by rhodopsin. The measurements were made by visual comparison with standards, but have since been confirmed by the precise spectrophotometric studies of Chase (68) and of Dartnall, Goodeve & Lythgoe (43). In common with other photochemical reactions, the speed of bleaching is uninfluenced by the temperature between 5° and 35° C. (65, 69). For the intact retina Kühne had already found that the photic bleaching rate is independent of temperature in this temperature range, and the observations have been confirmed by Amenomiyama (70). Finally the velocity of the photochemical bleaching in solution is directly proportional to the incident light intensity (71). All these data are such as would be expected of a primary photo-

process, and one may conclude that visual purple bleaching is such a reaction.

It is pertinent that many properties of vision point to the same conclusion, and that by assuming it is a fact, a theoretical description of these properties becomes possible (11). One instance out of many is the direct relation found between light intensity and velocity of visual purple bleaching. Arnold & Winsor (72) were able to show on theoretical grounds that this must be the case if Talbot's law for visual flicker is true, which of course it is.

A particularly revealing study of the photoprocess in visual purple bleaching has been made by Dartnall, Goodeve & Lythgoe (43, 65) who used the method of "photometric curves" (73) involving measurements of the absolute absorption of the solution and its changes with time. They find that the product of the quantum efficiency, γ , and the decadic extinction coefficient, ϵ , is constant for a given wave length. Their best results show this product to be $\epsilon\gamma = 2.3 \times 10^4$. Since temperature, intensity, and concentration of visual purple do not influence the basic velocity constant of the bleaching rate, the quantum efficiency is probably constant. Moreover, since light absorption by visual purple is also uninfluenced by temperature, the product, $\epsilon\gamma$, should be constant under a variety of conditions, and this turns out to be the case.

All these facts indicate again that the photoreaction is straightforward and not complicated by chain processes, and lead one to expect that the quantum efficiency is either 1 or less than 1. If γ is assumed equal to 1, the extinction coefficient is then $\epsilon = 2.3 \times 10^4$. Examination of available data shows that of the substances which absorb in the visual and near ultraviolet, very few indeed have an ϵ value above this. Should the quantum efficiency of visual purple be much less than 1, the resulting value of ϵ would then be exceptionally high.

A similar conclusion may be drawn from the study by Schneider, Goodeve & Lythgoe (26) on the value of $\epsilon\gamma$ for different parts of the spectrum. The product $\epsilon\gamma$ as determined by the bleaching rate for different parts of the spectrum varies very much as the absorption spectrum of visual purple. Since the photometric density is directly proportional to the extinction coefficient, ϵ , this also means that γ is constant even at different wave lengths, and strengthens the conclusion that γ is 1 or very near 1. Assuming it to be 1 gives ϵ a value of 2.3×10^4 for each chromophore. It is by the use of this value of ϵ that Broda, Goodeve & Lythgoe (42) have been able to determine the

carrier weight of protein for each prosthetic group in the visual purple molecule.

This value of ϵ is undoubtedly of the right order of magnitude, as the following computation shows. The vitamin A content of a bleached and faded bull-frog retina is 3.61 μg . (44). Dissolved in 1 cc. this amount yields a concentration of 1.3×10^{-5} moles per liter. The photometric density of the visual purple of one bull-frog retina dissolved in 1 cc. and measured through a depth of 1 cm. is 0.472 (23). If each molecule of vitamin A corresponds to one chromophore on the original visual purple molecule, we can determine the extinction coefficient, ϵ , for each prosthetic group from these two values. The equation is $\epsilon = d/ct$ in which d is the photometric density for a thickness t , and c is the molar concentration. This computation gives $\epsilon = 3.3 \times 10^4$, which is the same order of magnitude as 2.3×10^4 found by Dartnall, Goodeve & Lythgoe from completely different data and by wholly other methods. The agreement speaks strongly for the idea that the prosthetic group in the visual purple molecule is stoichiometrically related to one molecule of vitamin A.

Visual yellow.—When a dark-adapted retina is bleached by light, its color changes from rose red through chamois, orange, to pale yellow, and on prolonged irradiation to colorless. Much the same series of color changes occur in the bleaching of visual purple solutions, and Ewald & Kühne (74) were at some pains to show that this color series is quite different from that which appears when visual purple is merely diluted. In the latter case the color changes from rose red to pale rose and to lilac, and ultimately disappears in great dilution without ever showing any tinge of yellow. During bleaching, however, the yellow color is definite both in the retina and in solution. Kühne ascribed the yellow color to the appearance of a new colored product which he called visual yellow, whose presence he demonstrated by the increased absorption of a bleaching solution in the blue and violet at the same time that its absorption decreased in the green and yellow due to the disappearance of visual purple. The final colorless condition he attributed to a newly formed substance, visual white, which is responsible for the greenish fluorescence appearing in the ultraviolet when the retina is completely bleached.

For many years the existence of visual yellow was doubted largely because the absorption spectrum measurements of Koettgen & Abelsdorff (5) and the bleaching data of Trendelenburg (6) did not show it clearly. I can confirm this because in my earliest work with visual

purple (67) I had no difficulty in matching the color of a bleaching solution with mixtures of unbleached and bleached solutions. This is possible only when an imperceptible amount of new yellow color is formed during bleaching. However, Garten (7) obtained definite evidence of its existence by showing in a variety of ways that in the retina and in solution the absorption during bleaching rises in the blue while it falls steadily in the green and yellow. This has now been confirmed so frequently (21, 23, 68, 75, 76, 77) that there is no longer any doubt of its existence. In fact, it is now clear that not one, but at least two colored substances are formed when rhodopsin is bleached, and that the process involves several steps.

The simplest demonstration of this may be made by comparing the bleaching of visual purple at room temperatures and at 0° C. At room temperatures in daylight, the solution rapidly becomes pale yellow. The cold solution under the same conditions becomes orange. The orange color slowly changes at 0° C., but if the solution is brought to room temperatures, it rapidly becomes pale yellow. Lythgoe (21) has called "transient orange" the substance responsible for the orange color; and he has called "indicator yellow" the final product. Kühne probably did not differentiate between these two stages and his term "visual yellow" undoubtedly covers both stages; in fact, it is very likely that he never saw the transient orange stage because of its very transitoriness.

One of the main reasons for the historical difficulty in recognizing visual yellow lies in its sensitivity to pH; it is very pale in alkaline solution and strongly yellow and even orange in acid (68, 78). This is why Lythgoe has called it indicator yellow. Because of this property, visual purple when bleached in alkaline solution hardly shows any yellow color, but does so markedly in neutral and acid solution. Another reason for the difficulty concerns the temperature sensitivity of the reactions involved; this will be discussed below.

Dark reactions.—It is now known that the bleaching of visual purple involves more than the primary photochemical process. Fortunately, the photochemical experiments have nearly all been made under conditions in which the photoprocess is limiting, and thus correctly describe that process. Other reactions do take place, however. The first indication of complexity was the observation that after exposure to light, dried gelatin films containing rhodopsin (79) and even aqueous solutions (76) continue to lose color in the dark. This has been confirmed by Lythgoe (21) and the whole process has been

studied in some detail by Lythgoe & Quilliam (80) and by Wald (23). Though the evidence is still somewhat contradictory, the major outlines have been well established.

When exposed to very bright light for half a minute, a neutral solution of visual purple bleaches to an orange color. In the dark this orange color fades very rapidly to a yellow, and then much more slowly to a pale yellow, and occasionally becomes colorless. The fading of the orange color is so rapid that Lythgoe & Quilliam studied it in solutions kept at 3° C. so that the process is slowed down to perhaps twenty minutes.

Visual purple has its maximum absorption at 500 m μ . The newly formed orange color has its absorption maximum at about 465 m μ , so that immediately after the photochemical effect the absorption at this point is much higher than in the unbleached rhodopsin. At low temperature in the dark, this new band slowly decreases in height. However, at the same time the region below 410 m μ slowly increases and at precisely the same rate as the part above 410 decreases. The final result, even after an hour at room temperature, is a yellow substance whose absorption is much higher in the violet than that of either visual purple or the transitory orange stage.

There is no doubt that the first effect of the light is to produce the transient orange stage, which even in the dark then forms the yellow stage with indicator properties. Lythgoe regards these two steps as representing separate substances, transient orange and indicator yellow. It may be that they are stages in the separation of carotenoid from protein in the rhodopsin molecule. Wald (23) has found that retinas bleached by light at ordinary temperatures yield retinene at once to petrol ether, whereas retinas bleached in the cold yield little or no retinene at once, and only do so gradually. However, solutions of rhodopsin, even when completely bleached and faded, yield only a small fraction of their retinene content to petrol ether. The precise chemical meaning of the two stages is therefore still obscure.

Wald (23) has described several dark processes in the bleaching of visual purple solutions by light, but the data are not convincing. One of the changes involves the appearance of a rise in absorption at about 440 m μ simultaneously with a drop in absorption to either side. It just barely appears in neutral solutions and becomes increasingly prominent with increase in acidity. Its disappearance is slow, and is complete only in about twenty-four hours. This resembles Lythgoe's indicator yellow stage not only in its absorption spectrum, but in its

sensitivity to pH, and in its slow disappearance with time. There appears, however, a transient trace of maximum at about 480 m μ which may be related to Lythgoe's orange stage. Wald undoubtedly missed most of Lythgoe's transient orange step because he worked at 25° C.; at this temperature, it would be over in a very few minutes. Wald records another dark reaction, which is apparent in alkaline and neutral solutions, and which consists of a steady decline in absorption occurring maximally at 480 m μ . This is reminiscent of Lythgoe's transient orange, but seems much slower; perhaps it represents only its very last stages. Finally there is the slow general decrease in absorption throughout the spectrum first discovered by Hosoya (76), and shown best by freshly prepared solutions after bleaching by light. In most cases the absorption spectrum of completely bleached solutions resembles closely their petrol ether extracts.

The dark reactions often constitute a significant fraction of the total change in absorption which a solution of visual purple undergoes when exposed to light, and extractions vary widely in this respect. In some instances recorded by Wald (23) the decrease at 500 m μ after total bleaching was about 70 per cent due to light and 30 per cent to the subsequent dark reactions; in others, particularly in alkaline solutions, about 90 per cent of the change was photic and less than 10 per cent thermal. The relative proportions obviously depend on the wave length used in the measurements. The reaction products all have high absorption in the blue and violet. The fall in absorption is therefore least in the blue, and there are actually gains in absorption in the violet; the precise value of the wave length at which there is a transition from loss to gain in absorption depends on the pH.

Dichroic changes.—In addition to the photochemical and chemical changes already described for visual purple, there are some special ones found by Weigert which need to be briefly mentioned. It has long been known that thin layers of partially exposed silver chloride show color adaptation phenomena; they take on the color of the light to which they have been exposed for a short time. Weigert (81) found also that polarized light impresses its plane of polarization on such photochloride films, and that the specific color effects are greatest in the impressed direction of polarization. The result is a photo-dichroism which may be measured with precision. Further studies revealed that light-sensitive dyes embedded in gelatin or collodion films show similar dichroic changes, and Weigert (82, 83) has suggested that the color effects so produced may serve as a basis for color vision.

What is much more interesting is that visual purple embedded in gelatin films also shows photodichroism and color adaptation. First found by Weigert & Nakashima (79), these effects with visual purple layers have now been studied in considerable detail by Weigert & Morton (84, 85). After exposure to monochromatic light, photodichroism is produced only for wave lengths near the exciting one, with a maximum for the latter. Exposure to several portions of the spectrum, either simultaneously or consecutively, produces a dichroic effect with a maximum at a wave length whose color is the same as that of the color mixture of the exciting wave lengths. The maximum produced by white light is at 530 m μ and corresponds to the absorption maximum of visual purple in the dried gelatin layer. Exposure to mixtures of red and blue light, however, produces two maxima.

Results somewhat similar to these have been reported by Takamatsu (86) even for visual purple solutions. He finds that such solutions are optically active in that they can rotate the plane of polarized light. Exposure to ordinary light decreases this optical activity; exposure to monochromatic light produces a decrease which is maximal for that particular wave length. The color selectivity of this phenomenon is very sharp, and is greater in solutions made of visual purple from summer frogs than from winter frogs. White light also produces a maximum effect at 530 m μ , which wave length Takamatsu erroneously thinks is the maximum of the rod luminosity curve.

REGENERATION OF VISUAL PURPLE

In the retina.—In the living animal visual purple is constantly reformed as it is bleached by light, and under continuous illumination a balance is struck between the rate of bleaching and the rate of regeneration. If the animal is placed in the dark, the regeneration continues until a maximum concentration is obtained. There appears to be no limit to the number of times visual purple in the eyes of an intact animal may be bleached by light and regenerated in the dark.

The regeneration depends to a large extent on material supplied by the circulation, though the precise extent of the dependence varies with different species. The isolated mammalian retina, for example, shows practically no regeneration when bleached after removal from the animal. The isolated frog retina, however, is capable of considerable recovery in color (3). When bleached, it will regain some of its color in the dark, a process which may be repeated several times. Kühne showed that a retina which will no longer regenerate in the dark will

do so if it is replaced in the optic cup; evidently the material in the pigment layer may be drawn on for the necessary ingredient in forming visual purple. It is not the pigment itself which is responsible for this, because albino mammals regenerate visual purple quite adequately, as do animals whose retinas are backed by a tapetum (87).

The course of visual purple regeneration in the living animal was first observed by Kühne, and approximately measured by Gatti (32). The observations were extended by Abelsdorff (88) and by Garten (16), who has given a complete account of the earlier information. These observations did not record actual concentrations of visual purple in the retina; however, visual judgments in comparison with color standards were adequate to determine the time required and the effect of temperature on regeneration.

In recent years the subject has been studied more quantitatively; nevertheless the process is far from being described adequately. Fridericia & Holm (89) still compared the color of the retinas of albino rats after different times in the dark with color charts like those of Garten. They found that rats fed on a vitamin A-free diet had less visual purple and regenerated it more slowly than normally fed rats. Tansley (34) was the first actually to measure the concentration of visual purple in the retina during dark adaptation. She did this by removing the retinas and estimating their visual purple content spectrographically. She confirmed Fridericia & Holm's findings, and in addition plotted the course of the regeneration. Its kinetics may be best described by the usual monomolecular equation, though a bimolecular equation is not excluded.

Zewi (33) has made extensive regeneration measurements, using the frog; the concentration of visual purple was measured spectrophotometrically after extraction with digitonin. He confirmed Gatti's (32) work on temperature. The value of Q_{10} between 7.2 and 17.2° C. is 4.1; between 12.2 and 22.1° C. it is 2.8. I find that the data follow the Arrhenius equation fairly well and yield a thermal increment of very nearly 20,000 calories.

Zewi records that regeneration in the extirpated and opened eye is only about half as extensive as in the intact animal. Temperature apparently does not affect the rate of regeneration in the opened eye; this is an extraordinary observation and should be checked. Neither lactoflavin nor glucose has any influence on the regeneration of excised eyes; they are without influence in the living animal as well.

According to Zewi, oxygen is necessary for regeneration, because

excised and opened eyes regenerate more slowly and to a less extent in an atmosphere of nitrogen than in oxygen. This would seem to correspond to the observations of Jongbloed & Noyons (90) that in the dark, the posterior halves of frogs' eyes show a 20 per cent higher oxygen consumption than in the light. However, no such differences in oxygen consumption have been found by Warburg & Negelein (91), Kubowitz (92), Nakashima (93), and Oguchi (94). Most recently Chase & Smith (40) have reinvestigated this particular point and have also failed to find any increase in oxygen consumption concurrent with the regeneration of visual purple in half-eyes of the frog.

Solutions.—In 1878 Ewald & Kühne (74) reported that visual purple in solution, after being bleached by light, will regenerate some of its color in the dark. Though it is common knowledge that most investigators of visual purple vainly tried to repeat this observation, it was not confirmed until recently by Hecht, Chase, Schlaer & Haig (95). They were able to show that the newly formed material has the same absorption spectrum as the original visual purple. Regeneration occurs maximally at neutrality and falls off in acid and alkaline solutions. Its occurrence is most evident only after about 85 per cent of the original visual purple has been bleached away. However, Hosoya & Sasaki (96) have reported that under certain conditions one can observe regeneration in solutions which have been but slightly bleached.

According to Chase (97), the color of the light used for bleaching plays a deciding role in the amount of regeneration. Solutions bleached with yellow light regenerate hardly at all, while those bleached to the same extent with blue light regenerate to as much as 15 or 20 per cent of their original visual purple concentration. Solutions bleached by yellow light which then show no regeneration may be made to regenerate by exposure to blue light. After trying four different parts of the spectrum, Chase & Smith (40) find that the capacity for regeneration after bleaching by monochromatic light decreases steadily from violet to yellow.

It is to be noted that the extractive used also makes a difference in regeneration capacity. Solutions of visual purple in bile salts (95) and in digitonin (40) regenerate; those in sodium desoxycholate show no regeneration (20, 40). In careful studies on the effect of the pH, Chase & Smith (40) have delimited the regeneration range as between 5 and 9, with a maximum at 6.5. The temperature during the original extraction has no influence on the speed and amount of the subsequent regeneration.

The kinetics of the regeneration process have been studied by Chase & Smith (40). The course, at all wave lengths measured, follows a first order equation. This is true even for repeated regenerations by the same sample. These repeated regenerations give some insight into the nature of the reaction because on repetition the regeneration gradually fails. If this failure is due to the destruction of a catalyst, the second and third regenerations should be at a slower rate than the first, but should ultimately produce the same concentration of visual purple. If the failure is due to the disappearance of a reactant, the final amount of visual purple should be less for each successive regeneration. The latter is true, and is reminiscent of the behavior of isolated retinas on repeated bleaching and regeneration.

Chase & Smith followed the absorption spectrum of the solution during regeneration, and report that the newly formed material remains constant in color as it increases in concentration. If visual purple formation passes through a series of consecutive color changes similar to its bleaching, these are too rapid to be picked up at ordinary temperatures.

Several conclusions may be drawn about the nature of the material required for the regeneration of visual purple. It is a substance of small molecular weight, first because it is easily diffusible from the circulation and from the pigment layer, and second because there is only a trifling change in the molecular weight of visual purple on bleaching (25). If the substance is a product of visual purple bleaching, it is not an immediate one, because the bleached solution must first be changed by exposure to blue light before regeneration can occur. Its light-sensitive precursor absorbs in the blue, and is thus probably a yellowish material. A not entirely improbable guess is to consider it as related to either retinene or vitamin A, but the direct evidence is completely lacking. One must rely on the indirect information of the influence of vitamin A on vision.

Dark adaptation and vitamin A.—The last fifteen years have definitely established that vitamin A is the critically responsible agent in the well-known association of night blindness with dietary irregularities. The literature on this subject is voluminous, and has been adequately reviewed (98). The significant steps in the demonstration are few. Holm (99) first showed that night blindness in rats can be produced by a diet deficient in vitamin A, and the condition may be cured by ingestion of vitamin A. In the eyes of such A-deficient animals Tansley (34) found less visual purple than normally, and Fridericia

& Holm (89) showed that it is regenerated during dark adaptation more slowly than in normal animals. In human beings night blindness has been experimentally produced by an A-deficient diet (100 to 106) and the condition has been reversed by resumption of a normal diet.

Night blindness is a vague term and has to be replaced by quantitative statements about dark adaptation and visual thresholds. The visual threshold at any moment is the light intensity required just to elicit a visual sensation, and dark adaptation is the change which the visual threshold undergoes during a stay in the dark after an exposure to light. The change is enormous; after exposure of the eye to daylight, a stay of thirty minutes in the dark results in a decrease in the threshold by a factor of a million. The change is made in two steps, the first being mediated by the retinal cones, and the second by the retinal rods (107). The first is rapid and is over in three or four minutes; the second is much slower and takes about twenty-five minutes.

In most human subjects, the change to a highly A-deficient diet results in an almost immediate rise of the rod threshold. If the deficient diet is maintained, the rise continues until the threshold may be one hundred times as high as normal (104). Usually the rise is less than this, and the general condition is called night blindness. It is to be noted that on an A-deficient diet it is not only the final rod threshold which rises, but the final cone threshold as well (102, 108). In addition, the whole process of dark adaptation is slowed down.

Visual purple is the photosensitive substance responsible for rod vision, and the changes in dark adaptation are roughly paralleled by the changes in visual purple concentration. The mere fact that vitamin A affects both dark adaptation and visual purple concentration would not be enough to link vitamin A itself with the actual chemical regeneration of visual purple. However, if we add to this the existence of vitamin A in the retina and in the pigment layer (109, 110) and its appearance as an ultimate retinal product of the bleaching of visual purple, we are almost forced to the conclusion that vitamin A or some immediate product or modification of it is concerned with the chemical reaction which converts the light-insensitive protein into the light-sensitive visual purple during regeneration in the retina and in solution. The fact that vitamin A influences the cone threshold as well must be taken to mean that the visual pigment in the cones also probably contains a carotenoid as prosthetic group.

OTHER VISUAL PIGMENTS

Porphyropsin: Occurrence.—Kühne & Sewall (111) noticed that fish retinas look more purple than frog retinas. Bile salts extracts of fish retinas were shown by Koettgen & Abelsdorff (5) to have a difference absorption spectrum (unbleached minus bleached) whose maximum is at 540 m μ in contrast to one at 500 m μ found for all mammals, birds, reptiles, and amphibians. This position of the maximum was confirmed by Garten (7) for *Abramis*, and by Grundfest (112) for the sunfish *Lepomis*. As a result of these facts it has generally been supposed that there are two kinds of visual purple: fish visual purple and the usual visual purple from all other animals.

The situation became momentarily confused when Bayliss, Lythgoe & Tansley (113) found that for different species of fish the absorption maximum ranges between 505 and 545 m μ . But fortunately the matter has now been cleared up by Wald (114, 115, 116). There are two types of photosensitive material in fishes. One is the ordinary rhodopsin or visual purple, which, because its solutions are rose red, should have been called visual red as Boll first suggested, and which has an absorption maximum at about 500 m μ . The other is porphyropsin, so named by Wald since in solution it is really purple, which has a maximum near 522 m μ (22, 115).

The distribution of these two sensitive substances in fish is of great interest in evolution. Most permanently marine fishes have rhodopsin similar to that found in mammals, birds, and amphibians, whereas all permanently fresh-water fish and some marine fish which spawn in fresh water contain porphyropsin. Practically all fishes which migrate between fresh and salt water contain mixtures of rhodopsin and porphyropsin; the one which is ordinarily found in the fish's normal environment is always present in the greater concentration. The presence of either porphyropsin or rhodopsin does not depend on the immediate surroundings of the fish, but on the animal's genetic makeup.

Porphyropsin: Absorption spectrum.—Moderately pure unbleached porphyropsin solutions in digitonin from a variety of permanently fresh-water fish and some fresh-water-spawning marine fish have absorptions whose maximum varies 2 m μ to either side of 522 m μ . The band is distinctly unsymmetrical, going down toward zero in the red, but showing a minimum absorption in the blue-violet of more than half the maximum absorption. The precise location of this minimum depends on the species; it varies between 430 and 460 m μ . Be-

yond this minimum the absorption rises again into the violet; the ultraviolet absorption has not yet been studied. The absorption band of porphyropsin is somewhat wider than that of rhodopsin.

Wald (115) has shown that though the maximum of the unbleached porphyropsin solution is at 522, the maximum of its difference spectrum (unbleached minus bleached) is at 540; and this is precisely where previous workers had found it. Very likely those marine fish which Bayliss, Lythgoe & Tansley report as showing intermediate positions for the maximum of the difference spectrum may contain both rhodopsin and porphyropsin.

Porphyropsin: Chemical nature.—The nature of porphyropsin has been explored by means similar to those which have served for rhodopsin, and we owe our knowledge of porphyropsin to Wald (115).

In the retina the gross behavior of porphyropsin parallels that of rhodopsin. Isolated retinas of dark-adapted fresh-water fishes are deeply purple. Exposed to light they rapidly bleach to a russet hue which is maintained for hours at low temperatures. At room temperatures (25° C.) in the light the russet color slowly fades to colorless in three-quarters of an hour; in the dark some purple color is regenerated. The completely colorless retina does not regenerate in the dark.

Dark-adapted fresh-water retinas yield little or nothing to petrol ether. Retinas rapidly bleached by light turn russet or orange, and when extracted with petrol ether yield a substance which in chloroform is golden yellow, and which reacts with antimony trichloride to give a blue color. The absorption maximum of this blue-colored product lies at 705 m μ . In its behavior the yellow substance resembles the retinene from rhodopsin. However, with antimony trichloride the latter retinene gives a blue color whose absorption maximum is at 664. To differentiate the two, Wald has called retinene₂ the product from porphyropsin, while retinene₁ is the product from rhodopsin. In chloroform solution retinene₁ has an absorption maximum in the ultraviolet at about 385 m μ , while retinene₂ has a maximum at 405 m μ . Retinene₂, like retinene₁ from rhodopsin, changes color with hydrogen ion concentration; it is very light yellow in alkaline and deep orange in acid.

When fresh-water retinas are left at room temperatures after bleaching they gradually become practically colorless. They then no longer yield retinene₂ to petrol ether, but give instead a substance which when tested with antimony trichloride produces a blue color whose maximum absorption is at 696 m μ and whose existence had

not been known before. No trace of ordinary vitamin A appears in such fresh-water retinal extracts.

This new substance was almost simultaneously discovered in certain fish livers by Lederer & Rosanova (117), and was shown by Gillam, Heilbron, Jones & Lederer (118) to be a homologue of vitamin A containing an additional ethylene group. It has many functions similar to vitamin A, and because it replaces vitamin A in the cycle of fresh-water fishes Edisbury, Morton & Simpkins (119) have called it vitamin A₂. Its chemical properties are now well known as well as its distribution in nature (114, 120). In chloroform it shows an absorption band similar to that of vitamin A (which may be called A₁) but located at 355 mμ instead of at 328 mμ as for vitamin A₁.

In solution and in the retina porphyropsin behaves much like rhodopsin toward various treatments and reagents. Wald reports that it does not diffuse through a wet collodion membrane and may be dialyzed. It is not precipitated by half saturation with ammonium sulfate, but does precipitate at about 0.8 saturation. Its color is very rapidly destroyed by normal solutions of potassium hydroxide, hydrochloric acid, or sulfuric acid, by strong ammonia, by methyl and ethyl alcohols, chloroform, acetone. The color is destroyed at high temperatures, and the temperature coefficient of the destruction is high.

All this information indicates that porphyropsin is probably a protein. Its color, as well as its yield of carotenoid on bleaching, shows it to be a carotenoid-protein much like visual purple. The one important difference between the two substances is in the position of the absorption maximum by about 22 mμ. This corresponds to the difference of about 18 mμ in their retinenes, and of about 27 mμ in their vitamins A, all differences being in the same direction. The shift in absorption maximum is most likely brought about by the presence of the extra ethylene group in the porphyropsin carotenoid, since such an addition in an homologous series of polyenes shifts the absorption maximum 20 to 27 mμ toward the red (121). The added ethylene hardly affects the chemical behavior, and thus accounts for the remarkable similarities in the behavior of porphyropsin and rhodopsin. As Wald points out, this is not without its importance for understanding the ultimate chemical basis of color vision (122).

Cone substances.—Rod vision and cone vision are essentially similar in behavior; under a variety of conditions, the kinetics and dynamics of the two sensory systems are alike, and may even be described by the same equations (11). Such functional similarity

argues for structural similarity in their chemistry, and indeed the similar effects of vitamin A on the two systems (102) strengthens the argument.

From the chemical viewpoint, the essential differences between rod and cone vision reside in their spectral and brightness sensibilities. The region of maximum spectrum sensibility for the cones is at 555 m μ , for the rods 500 m μ . Different substances are therefore probably concerned in their photoreception. Moreover, at their spectrally maximal sensitivity, the cones require 50 to 100 times more energy for a minimal stimulus than the rods do at theirs. If the two materials are similar in their capacity to absorb light, then the photosensitive substance in the cones is about one one-hundredth the concentration of visual purple in the rods. To find sensitive pigments in the cones, one must thus look for very slight photochemical effects in the region of 555 m μ .

The retina of the turtle contains cones only; therefore, it has been used in the search for the cone substance. Spectrophotometric examination of a glycocholate extract of such retinas by Koettgen & Abelsdorff (5) failed to show any difference between the unexposed and the light-exposed solution. On the theory that a thick layer of solution would compensate for its low concentration, Garten (16) observed visually an extract 6 cm. thick, but could see no change in color as the result of exposure to light. Similarly, von Studnitz (123, 124) found no difference with the same material. These failures are not unexpected, because the instruments used were not sensitive enough. However, Hosoya, Okita & Akune (125) have recently reported that even visually they could observe a photolabile reddish color in glycocholate extracts of turtle retinas. Moreover, the difference spectrum of the solution before and after exposure to white light shows several absorption peaks, only one of which (at 460 m μ) can be considered well defined.

Because retinal extracts yielded no results, von Studnitz (123, 124, 126) investigated the isolated retina itself. From measurements with the Pulfrich Stufenphotometer he reports among other things that turtle retinas possess a photosensitive substance whose absorption maximum is near 560 m μ , that it is destroyed by light fourteen times faster than visual purple, that it is insoluble in glycocholate and digitonin, that it is soluble in ether and can be bleached in ether solution, and that it regenerates in the dark. However, careful study of his two papers reveals many errors in method and in treatment of

measurements, and leaves one in doubt about the conclusions. Two examples will suffice, one general, the other specific. Throughout, percentage absorptions are added and subtracted as if Bouguer, Lambert, and Beer had never existed. Specifically, the dark-adapted retina, as measured, turns out to be nearly opaque—a fact in itself remarkable—and the minor differences in opacity along the spectrum are used to characterize the cone substance as having a maximum absorption at 560 $m\mu$. The actual absorptions at 540, 560, 587, and 670 $m\mu$ are given as 97.49, 98.71, 97.22, and 98.24 per cent respectively, but there is no evidence that the differences among the values are beyond the experimental error. The report that the ether extract is photolabile has not been confirmed by Hosoya, Okita & Akune (125).

It is clear that the cone substances must be looked for only with very sensitive instruments. Fortunately, spectrophotometers are now available (113, 127, 128) with a sensitivity between ten and one hundred times greater than those of years ago. With such an apparatus Wald (129) has reported finding a photolabile cone substance in the digitonin extract of chicks' retinas. The chick's retina contains many more cones than rods, and its digitonin extract shows the presence of visual purple. Because cones are more sensitive to extreme red light than rods, Wald exposed the digitonin extract to light beyond 650 $m\mu$, and measured its absorption before and after exposure. The result is a difference spectrum which is roughly symmetrical to each side of a maximum absorption at 575 $m\mu$. When red light produced no further change, exposure to white light resulted in two effects, a change whose difference spectrum has a maximum at about 508 $m\mu$, and an increase in absorption of the extract as a whole at about 430 $m\mu$. Both these effects are characteristic of visual purple bleaching. The two difference spectra correspond roughly to the spectral sensibilities of the chick (130) after light adaptation (cone vision, maximum at 580 $m\mu$) and after dark adaptation (rod vision, maximum at 520 $m\mu$). In the chick retina cones are much more numerous than rods. Yet in the extract the photometric density of the cone substance is less than of visual purple. This speaks again for the comparatively low concentration of the sensitive substance in the cones.

There have been no published confirmations of Wald's findings. However, in our own laboratory Mr. A. F. Bliss has reproduced Wald's work with good agreement. Moreover, Chase (131) has shown that even in ordinary extracts of frog retinas one finds a photolabile sub-

stance with an absorption maximum at 530 m μ which can be bleached away by exposure to extreme red light. Subsequent bleaching with white light shows the usual visual purple difference spectrum. Similarly Hosoya, Okita & Akune (125) have found that extracts of toads' retinas prepared in blue light differ from those prepared in red light by an absorption showing a fairly well-defined maximum at 550 m μ .

All these results must be considered as significant preliminary trials. Before the cone substance or substances can be considered as well established, it will be necessary to introduce greater stability in the procedure and greater refinement in the measurements so that their absorption spectra will be as easily reproducible as those of visual purple.

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THE CHEMISTRY OF MUSCLE

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*General summary.*¹—The discovery of striking interactions between myosin and adenosinetriphosphate gives promise of a much more complete theory of muscular contraction than has yet been attempted. Most of the recent work on muscle has been devoted, however, to a reanalysis of earlier theories in the light of new experiments. Two newer hypotheses, the citrate cycle and the "direct oxidative" theory, have gained some heavily contested ground, but are not yet on an assured footing. The discovery that resting and activity metabolisms can be separated by azide shows that important findings can still be made by classical methods; yet the search is becoming ever more intense for techniques capable of following the time course of specific chemical reactions associated with muscular contraction.

Structure and muscle proteins.—Astbury & Dickinson's work on the x-ray structure of myosin and of muscle (1) has appeared during the period under review, but it has been so adequately dealt with both in *Annual Reviews* (2) and elsewhere that the briefest summary must suffice here. Myosin was found to give x-ray patterns remarkably similar to those of wool and hair, and to undergo the same modifications upon stretching. The alpha-beta transformation could be clearly shown in both systems, and the previously developed interpretation of polypeptide chains assembled in regularly spaced grids of fixed transverse dimensions but able to assume either a straight chain (beta) form or a once-folded (alpha) form was extended to include

¹ It is four years since the chemistry of muscle was treated in the *Annual Reviews*, Dr. Parnas having been prevented by the war from making his expected contribution two years ago. It would be difficult for anyone, and is certainly beyond the capabilities of the present reviewer to discuss adequately the flood of work on muscle which has appeared in these years. I have deliberately restricted this paper to a narrower range of topics than have been treated heretofore, transferring as much of my task as possible to the shoulders of other reviewers, whose contributions are separately listed at the end of the bibliography. Fortunately, general articles of this kind have been both plentiful and excellent.

myosin as well as keratin. The double-folded (supercontracted) form, in which the orientation of transverse spacings was largely lost, could also be demonstrated in both classes of protein. The x-ray photographs of intact muscle reveal the alpha type, which is retained even on stretching, the myosin molecules remaining folded, though the chains become better oriented. The picture of contracted muscle resembles those of supercontracted myosin and keratin, and corresponds to a deranged space lattice. Thus muscular contraction is probably associated with a weakening of the transverse bonds which tend to keep the grids in place, coupled with additional folding of the polypeptide chains. Several details of Astbury's polypeptide grid theory have been regarded with skepticism by other workers. In order to make room for the side chains and to obtain better agreement with observed spacings, the manner of folding the alpha chains has just been radically revised (3), the folds being now roughly rectangular instead of hexagonal. It is apparent that this chapter is by no means closed.

The significance of disulfide groups in these transverse bonds has not been fully elucidated: the failure to find any parallelism between changes in the number of free sulfhydryl groups in myosin treated with various denaturing agents and alterations in birefringence of flow (4, 5) presents a difficulty in interpretation. Many mild agents quickly reduce birefringence without producing any significant change in the number of sulfhydryl groups as indicated by porphyrindin titration, but some substances (glycine, for example) affect these two properties in the reverse order. A close connection was found between flow birefringence and viscosity of myosin solutions, as was expected, since both are reflections of molecular asymmetry.

Myosin and adenosinetriphosphate.—It has been shown by Engelhardt & Ljubimowa that myosin, or some substance at present indistinguishable from it, acts enzymatically in the dephosphorylation of adenosinetriphosphate (6). The effect was obtained with muscle extracts containing the water-insoluble proteins, and it was destroyed by myosin-denaturing agents. Myosin appears only to break down the triphosphate to diphosphate; a diphosphatase action is associated with the soluble muscle proteins. This enzymic action of myosin has been confirmed in two laboratories (7, 8). Needham, Shen, Needham & Lawrence have reported that when potassium adenosinetriphosphate is added to a myosin sol, its birefringence rapidly falls to about two thirds its initial value. The effect is not obtained with

either potassium adenylate or potassium pyrophosphate; it is reversible, returning in two or three hours to its original value. Contrary to the findings of Edsall & Mehl (5), they found that the reversibility of myosin birefringence could also be shown after treatment with lithium, calcium, urea, etc. The adenosinetriphosphate effect, however, was obtained with only one tenth the molar concentration required by these other substances. Needham *et al.* (8) suggest the following tentative theory for muscular contraction. In resting or recovering muscle adenosinediphosphate accepts phosphate from phosphocreatine, phosphopyruvate, or glycerophosphate, thereby becoming adenosinetriphosphate. It then acts as a phosphate donor to myosin, the phosphorylated myosin being in the extended form. On contraction the extended myosin phosphate liberates inorganic phosphate and contracts. Many points in this theory obviously need elucidation and confirmation. The energy relations involved are still untouched, and direct evidence for the phosphorylation and dephosphorylation of myosin, suggested by Kalckar (9), is urgently needed. The theory's great significance lies in the fact that it is the most promising attempt yet made to bridge the yawning chasm between metabolic processes and changes in structural organization accompanying activity.

Anaerobic processes in muscle.—Recent progress in the study of anaerobic carbohydrate breakdown *in vitro* is outside the scope of this review.² It is generally held that the chain of reactions whereby phosphate groups are shuttled back and forth between carbohydrate degradation products, creatine, and adenylic acid, which have been discovered in enzyme extracts and preparations of yeast or muscle, also applies to the living tissue. This view has, however, been repeatedly challenged by Sacks, whose views have been summarized in his 1941 review. He believes that the primary energy-yielding reaction for anaerobic contraction is the breakdown of glycogen to lactic acid without intermediate transfer of phosphate groups from either phosphocreatine or adenosinetriphosphate. When this mechanism is inadequate, hexose monophosphate is formed from glycogen and phosphocreatine. Hydrolysis of phosphocreatine serves only to neutralize the

² The most extensive recent review known to the writer is J. K. Parnas' article *Glycogenolysis* in the *Handbuch der Enzymologie* (1940). Two general articles in English by C. F. Cori and O. Meyerhof are in *Biological Symposia* Vol. V (1941).

lactic acid formed. Recent evidence brought forward in support of this view is twofold. First, several of the extract reactions cannot be demonstrated in intact muscle. For example, no triosephosphate could be found in iodoacetate-poisoned frog muscle in rigor, even in the presence of ample quantities of its precursor, hexosediphosphate (11). Second, experiments with radioactive phosphorus (12) appear to show that the labeled phosphate groups show much less chemical mobility than would be predicted from the accepted schema in which phosphate groups are passed back and forth. There is some question, however, whether the published data are sufficient definitely to exclude the participation of adenosinetriphosphate and of phosphocreatine in the formation of lactic acid.

Aerobic metabolism in mammalian muscle.—Flock, Ingle & Bollman (13) measured the changes in the content of labile constituents of rat gastrocnemius muscles before and during steady state activity with the circulation intact. The muscles were maximally stimulated three times per second, the rate of work production declining to a steady state during the first minute of stimulation from an initial value about twice as high and thereafter remaining constant for long periods. For chemical measurement the muscles were suddenly frozen, fragmented, and analyzed. During the first minute of work there was a rapid accumulation of lactic acid and hexosemonophosphate, a somewhat slower increase in inorganic phosphate, a rapid decline in phosphocreatine and glycogen and a delayed decrease in adenosinetriphosphate. In the subsequent fifteen minutes of continued activity the lactic acid and hexose monophosphate returned to their resting levels, there was no significant change in the phosphocreatine or glycogen contents, and there was a very gradual return of inorganic phosphate and adenosinetriphosphate to resting values. The authors concluded that since continued aerobic contraction produced little further change in the concentrations of the labile substances, they are mainly involved in the early phases of work and participate but little in steady state activity. It should be pointed out, however, that the observed course is exactly what would be expected if these substances functioned as intermediate energy carriers in normal aerobic activity, for if resynthesis follows mass action principles, the average or "equilibrium" concentration would be shifted in going from rest to steady repetitive activity. Any technique which measures only the change in total amounts of metabolites with a time uncertainty at least as long as the period between one contraction and the next can only be expected to

measure changes in this "equilibrium" and not in the rates of breakdown and resynthesis.

The restitution of glycogen following a steady state of work in the same preparation has also been studied (10), and was found to be much slower than the removal of lactic acid. Furthermore the rate of restitution was not influenced by the presence of lactate, by epinephrine, or by insulin hypoglycemia, but was somewhat increased by the presence of glucose. It only occurred with the circulation intact. It appears that the slowness with which it is resynthesized prevents it from playing a major role as a fuel for steady state activity.

The resynthesis of phosphocreatine and adenosinetriphosphate in intact cat muscle following five minutes of repetitive activity has been studied by Sacks (14). With the circulation intact, 47 mg. or about two thirds of the phosphocreatine was hydrolyzed during stimulation. In the following five minutes of recovery only half of this was resynthesized. Sacks has calculated that if all the energy of contraction came from phosphocreatine breakdown, according to Lundsgaard's original hypothesis, the average hydrolysis rate during activity would be about ten times the average resynthesis rate during recovery, and he concludes that the cycle is too slow to account for a major portion of the energy transfer in a steady state. The slow resynthesis of phosphocreatine fails also to agree with the rapid oxygen consumption of stimulated muscle. In order to produce a measurable breakdown of adenosinetriphosphate by stimulation, it was necessary to clamp the circulation, thus producing partial anaerobiosis. During subsequent recovery, the circulation was left unclamped, yet the resynthesis of adenosinetriphosphate was even slower than that of phosphocreatine. It was concluded that this reaction also plays no important part in aerobic contraction.

The kinetic argument, as applied here by Sacks, makes one assumption which should be emphasized. It is that the rate of recovery as measured during the time between the freezing of the control muscle and that of the test muscle is of the same order as that taking place between successive contractions in a steady state. Should the resynthesis immediately after contraction be very rapid and then fall off to a much lower rate, as was indicated in the transparency changes measured by von Muralt in frog muscle, the argument would not apply—a considerable amount of recovery might have taken place between the last contraction and the freezing. This possibility would be excluded if the residual phosphocreatine immediately after stimulation

were found to be very low. In Sacks's experiments, however, it was almost a fourth of the resting value.

The citrate cycle in aerobic metabolism.—The citrate cycle as developed by Krebs and his colleagues will not be treated in this review. The subject has now become so important that it occupies considerable space in every survey of biological oxidations. But as yet there have been almost no attempts to apply the system, the details of which have been worked out almost exclusively on minced muscle, to the intact muscle. The relation of the cycle to activity and resting metabolisms, the time relations of the successive steps as a guide to their possible roles in contraction, the analysis of free energy changes in different reactions as a clue to possible suppliers of energy to the contracting system, all these problems are still completely unexplored.

Separation of "resting" and "activity" metabolisms of frog muscle.—Stannard's important discovery that the activity consumption of frog muscle can be inhibited by means of azide without affecting the resting consumption (15) has been confirmed and amplified (16, 17). The effect was equally definite whether the activity was that of normal contraction, of contracture induced by potassium chloride, acetylcholine, or a high concentration of azide, or by the chemical stimulus of caffeine in subcontracture doses. It thus appears that caffeine continuously releases metabolic processes normally set off by electrical stimulation, as previously hypothesized by Meyerhof and by Hartree & Hill. One molecule of azide appears to inactivate one enzyme group, as indicated by the relation between inhibition and concentration of inhibitor. Glycolysis production is not inhibited by azide. Fluoride, arsenite, and the "copper-inhibitors" behave qualitatively like azide (17). Activity oxygen consumption thus passes through the Warburg-Keilin system, which is "thrown into circulation" as a result of the contraction, either by removing some inhibitor of cytochrome oxidase present in resting muscle, or by providing substrate. The maximum rate at which activity oxygen consumption can proceed is probably dependent upon the concentration of cytochrome oxidase (16). Corresponding experiments have not been reported on non-amphibian muscles.

The curious ability of frog muscle to oxidize carbon monoxide has been confirmed (18). This poison may act in two different ways on the tissue, first to inhibit a portion of the resting metabolism, and second to provide an additional substrate for "activity" metabolism. Since carbon monoxide is neither a normal substrate nor an inter-

mediate member of any known metabolism chain, the significance of this behavior is a mystery.

Oxygen consumption of resting dog muscle.—Pappenheimer, using a limb-lung preparation, has been able to follow continuously the resting oxygen consumption of isolated dog muscle, the oxygen content of the blood being measured photoelectrically (19).

By keeping the blood flow constant and varying the oxygen saturation of the arterial blood, Pappenheimer was able to measure oxygen consumptions at various oxygen tensions. He found the unexpected result that there was a significant falling off in oxygen uptake when the oxygen tension in the venous blood fell below 55 mm. Hg. (85 per cent saturation), but remained at about two thirds of its maximum value when the tension was anywhere between 20 and 50 mm. Hg. This suggests that about two thirds of the resting respiration passes over an enzyme system which is saturated at an oxygen tension of 20 mm. Hg. or below, while the other third uses an enzyme system with very much lower affinity.

When the vasoconstrictor nerves were stimulated there was an "apparent oxygen debt" which was only partially repaid when the circulation returned to normal (20). Since resting oxygen consumption may be of a different type from activity respiration, the significance of these results for active metabolism is obscure. Corresponding experiments with stimulated muscle are eagerly awaited.

The time course of oxygen consumption in active muscle: Mammalian muscle at 37° C.—Kramer and his colleagues (21, 22) have followed the oxygen consumption of the gastrocnemius muscle of anesthetized dogs, measuring continuously both the oxygen saturation and the blood flow, by means of Kramer's *Sauerstoffuhr* and Anrep's hot wire anemometer respectively. The continuous registration of both blood flow and the arteriovenous oxygen difference enabled these workers to obtain a much more complete picture of the oxygen conditions during and after work than was previously available in mammals. During tetanus they found that even the first spurt of blood that came from the muscle at the start of contraction was more reduced than the resting venous blood, from which they concluded that the rise in oxygen consumption was instantaneous or at least as rapid as the time lag inherent in their method (2 to 4 seconds), thus agreeing with measurements of the initial speed of reduction of intracellular myoglobin in the cat's soleus. This conclusion can hardly be drawn from these experiments, as Pappenheimer (20) has shown that considerable

quantities of blood may be statically pooled in the capillaries of resting muscle. The first rush of blood on contraction represents primarily this "reservoir of anoxemia," and not an instantaneous increase in oxygen consumption. The major portion of extra oxygen consumed as a result of a short tetanus was found to be taken up after contraction was finished. At least a part of the delay, however, was found to be due to inadequate blood and hence oxygen supply to the tissue, for the oxygen consumption in the earlier portion of a long tetanic contraction could be markedly increased by dilating the blood vessels with an injection of acetylcholine shortly prior to the contraction.

The time course of oxygen consumption in active muscle: Frog muscle at 0° C.—The time course of oxygen consumption of frog muscle has been very carefully measured gasometrically by D. K. Hill (16). By working at 0° C. where metabolic processes are very slow, he was able to avoid much of the ambiguity caused by diffusion time lags inherent in any two-phase method.³ The muscle was fully supplied with oxygen throughout. In every case practically all of the extra oxygen due to activity was taken up long after the contraction was complete. Thus, after a twelve-second tetanus, the extra oxygen was only half consumed in about ten minutes, whereas for a one-minute tetanus, it took twenty minutes for half consumption. This shows unequivocally that under these conditions the "activity" oxygen can only be used for recovery purposes. No "direct oxidative" mechanism is compatible with the experimental data.

Muscle heat.—It has also been shown by D. K. Hill that the time course of recovery heat of frog muscle at 0° C. following a short tetanus very closely parallels that of the oxygen consumption of muscles identically stimulated (16, 23). This was true both at pH 6 and at pH 7.2, where the shapes of the curves differed markedly from each other. This would indicate a rigid coupling between the reversal of the anaerobic energy-yielding reaction, which Hill assumes to be entirely phosphocreatine breakdown, and the oxidative process which drives it.

³ The correction for diffusion usually amounted to about 25 per cent of the measured time for half-consumption of extra oxygen. The calculation assumed standard values for the diffusion coefficient of oxygen in animal tissues, and made no additional correction for additional delay introduced by gas-liquid interface. The delay would have to be very large indeed to affect the qualitative conclusions of the experiments. In the analogous case of carbon dioxide, it could be controlled, and was found to be negligible.

A. V. Hill (24) has reinvestigated the aerobic recovery heat of frog muscle at 0° C. under a variety of conditions, and has found that the ratio of total energy to initial energy is consistently about 2 whether it is measured for a single contraction with complete recovery, or for a long series of contractions in a steady state, where the recovery heat is measured between stimuli, and where it may be several times as great as the resting metabolic rate. Sacks's suggestion that "post-stimulation heat merely represents the inertia of the tissue in returning to the resting metabolic level after a burst of activity" (10, 14) is thus rendered very improbable, at least for this tissue at 0° C.

A. V. Hill's (25) analysis of the regulation of energy liberation by muscle, based on very careful measurements of the extra heat liberated by a muscle on shortening, has provided new kinetic limitations which must ultimately be applied to the series of chemical reactions furnishing the energy for contraction. In its present form, however, the theory requires no assumptions as to specific chemical mechanisms, and falls outside the scope of this review. If found to be generally applicable to muscle, its implications for muscle biochemistry are portentous.

In the heyday of the classical lactic acid theory of muscle, the different phases of muscle heat were ascribed to specific chemical reactions with a degree of confidence which later work has not always justified. It must not be forgotten, however, that the limits prescribed by the thermal data (in so far as they are adequately controlled) are inexorable, and that no theoretical mechanism can survive which violates them.

The time course of pH changes in muscle.—Two noteworthy studies have been made of the pH changes which accompany and follow muscular activity and of the probable chemical processes which they indicate, one by Dubuisson using a special glass electrode with a very short time of registration, the other by D. K. Hill using his volumetric technique to follow carbon dioxide exchange (27). Both followed the changes continuously; both reduced the uncertainties due to time lags in measurement by slowing up the muscle reactions as much as possible, one by using smooth muscle instead of striated, the other by working at 0° C. The glass electrode method was used to observe reaction changes during and immediately after contraction while the volumetric one was rapid enough only for postcontractile phenomena but followed them for long periods with very small shift

in base line. The results, in so far as they can be compared, are of similar import. Since there is less ambiguity in the interpretation of the later phases than in the earlier ones, the series of reaction changes will be discussed in chronologically reverse order. Both workers agree in finding a large, slow, long-continued acid drift after stimulation, which outbalanced earlier changes, in all experiments in which oxygen consumption was inhibited and lactic acid formation was not. If the muscle were poisoned with iodoacetate (glass electrode) or if it were kept at a pH of about 6, where no lactate is formed (volumeter), this last phase disappeared; it is therefore an indication of lactic acid formation. Preceding this was a phase of increasing alkalinity and recovery which both authors attribute to phosphocreatine hydrolysis and resynthesis; this conclusion was supported in Hill's work by the observation that the carbon dioxide absorption could be much reduced by working at pH 9 where the hydrolysis of phosphocreatine has been shown to cause no reaction change. In Hill's work, by far the largest part of this phase occurred after the contraction was over which would definitely preclude this reaction from supplying the immediate energy for contraction of the frog gastrocnemius. This possibility could not be eliminated in the frog stomach muscle studied by Dubuisson, where this phase reached its maximum at the onset of relaxation. The volumetric technique under the conditions used was not able to detect earlier and more rapid changes than these, but the glass electrode was able to show that they were preceded by two more, an acid one immediately preceding that due to phosphocreatine and a small alkaline one immediately following stimulation; the former was ascribed by Dubuisson to the breakdown of adenosinetriphosphate, while the latter was of unknown significance.

Lactic acid production in intact muscle.—There is now a considerable measure of agreement about the conditions for the appearance and disappearance of lactic acid in the muscle with intact circulation, and of its interpretation. The general character of Bang's (28) findings on man have been confirmed and amplified in several different preparations. At the onset of a period of steady state activity, there is a fairly rapid rise of lactic acid production, which ceases at about the time that circulatory and respiratory adaptations to increased activity are complete. Following this, whether work is continued or stopped, there is a gradual reduction of lactic acid content. This has been found by Bang for man (28) and by Kramer *et al.* for dog (29), in experiments in which blood lactate was used as an indicator, though there

has been some disagreement as to how the magnitude and time course of the effect were modified by possible diffusion barriers to lactate from muscle to blood. Newman (30) determined the distribution of lactate between the blood and muscles of rats before and after stimulation, and found the increments to be equal, from which he concluded that the blood concentration did reflect correctly the muscle value. This was in agreement with the earlier findings of Margaria, Edwards & Dill, and in contradiction to those of Sacks. The uncertainty does not exist in the experiments of Flock *et al.* (13) who suddenly froze rat muscles after various amounts of work and recovery, and analyzed them subsequently. The rapid rise in lactic acid during the first minute was followed by a gradual decrease to the resting level as work continued. The rate of this return was independent of whether work continued or not; it was not accompanied by a restitution of the depleted glycogen stores. The results are in general agreement with previous work on cats by Sacks.

The generally accepted interpretation of these changes is that at the start of exercise the muscles are operating anaerobically and have to draw upon lactic acid formation for their energy of contraction; once circulatory and respiratory adjustments have been made, so that the oxygen supply is adequate, no further lactic acid is formed, even as an intermediate, and that already formed diffuses into the blood stream. This conclusion is supported by D. K. Hill's measurements on frog muscle at 0° C. (27). He found that the lactic acid formation in a muscle poisoned by cyanide was much slower than the oxygen uptake in the corresponding unpoisoned muscle. If lactic acid formation were a part of the normal aerobic cycle, it would have to be both formed and removed as rapidly as oxygen was taken up. The evidence has thus become increasingly satisfactory in the last few years that the formation of lactic acid is not an intermediate step in normal aerobic contraction, but that this reaction does provide the chief source of energy, though not the only source, in muscles inadequately supplied with oxygen.

The fuel for muscular exercise.—Gemmill, in his review, "The Fuel for Muscular Exercise," which has just appeared sums up the evidence which has been obtained from studies on the effect of diet on efficiency and on prolonged exertion, on the respiratory quotient during exercise, on the changes in blood carbohydrate, and on the output of nitrogen in the urine. He concludes that while carbohydrate is of first importance as a direct fuel, the indirect utilization of protein or

fat must be an efficient process since high muscular efficiencies can be obtained even on exclusive diets of these substances. Gemmill was unable to obtain experimental evidence that fat was oxidized directly by the muscle, as the fat content of phlorhizinized rat muscle showed no decline in fat content on being stimulated to exhaustion, though there was a marked drop in glycogen (31). He could not, however, exclude the possibility that fatty substances in the blood were oxidized in the muscle. Furthermore, it has recently been shown quite definitely that the aglycemic rat heart can utilize fatty acids from both blood and heart muscle (32). There is no doubt that fat can provide practically the sole overall fuel for exercise, which means that it must either be burned directly in the muscles or transformed to carbohydrate or ketone bodies in the liver, being then transported in this form to the periphery. Stadie (33), in a review of fat metabolism in which he discusses the detailed evidence, concludes that the conversion of fat to carbohydrate in the liver is doubtful and that the partial oxidation therein of fats to ketone bodies would require a considerably greater oxygen uptake by this organ than has been observed or is probable. He states: "the extreme view, held by some physiologists, that carbohydrate only can be utilized by muscle appears no longer tenable." Acetone bodies disappear in large quantities when perfused through stimulated muscle (34); active muscle appears able to use ketone bodies as a fuel.

"Classical" and "direct oxidative" theories of contraction.—Two divergent trends of opinion in muscle biochemistry are summed up in two recent reviews by Meyerhof and Sacks. Common ground is found on many points: muscles can contract anaerobically and, if they do, energy is obtained from the formation of lactic acid, while phosphocreatine disappears, hexose monophosphate and inorganic phosphate appear, and adenosinetriphosphate is hydrolyzed. Furthermore, it is agreed that the final viscous-elastic effector mechanism is the same, whether the muscle contracts aerobically or anaerobically. The questions at issue may be stated as follows: How far back in the chain of processes leading to contraction do the two types of activity converge on their "final common path"? Do all the anaerobic processes take place in the presence of oxygen just as they do in nitrogen, later to be reversed by oxidative processes, or are some of them forestalled? The extreme form of the "direct oxidative" theory says that all of them are forestalled. The evidence of the last few years favors neither extreme. It is now hard to resist the conclusion that in normal

aerobic contraction lactic acid formation need not take place, even as an intermediate. On the other hand, it is equally hard to avoid the conclusion that oxygen enters to restore a system broken down essentially in the same way whether oxygen is present or not. Do the dephosphorylations of creatinephosphate and of adenosinetriphosphate play a role in this system or not? The principal argument against accepting them is the kinetic one: they won't shuttle back and forth quickly enough to account for the overall activity of the muscle! However, our methods for determining this have been none too certain. The Achilles heel of our attack has been our inability to follow unambiguously the time course of local chemical changes during and after the period of contraction. The certainty of our answers will reflect our progress in the development of these methods.

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AVIAN BIOCHEMISTRY

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The field of avian biochemistry has not previously been treated in the *Annual Review*. The literature is vast; the economic importance of poultry has caused an intensive investigation of their nutrition, genetics, physiology, and embryology. The ease with which chicks may be procured and maintained in the laboratory has led to a widespread use of chickens as experimental animals, and this also has resulted in much information concerning the species. Consequently, it is possible to review in this article only a few sections of the assigned topic. Many important fields have been completely omitted, and the treatment of other material has been biochemical in a restricted sense of the word. Most of the literature reviewed is of recent date. Reviews of various fields related to avian biochemistry have appeared elsewhere (1 to 6).

AMINO ACID REQUIREMENTS AND METABOLISM

Critical studies of the amino acid requirements of the chick have only recently become feasible. Long after it was found possible to maintain and grow rats on highly purified diets, the more complicated nutritive requirements of the chick could not be met except by the use of natural feedstuffs. The chick is very sensitive to the lack of a number of dietary factors, including vitamin K, choline, manganese, potassium, glycine, arginine, an essential carbohydrate, and certain unidentified substances in yeast. With the provision of these factors in protein-free forms, it has become possible to conduct experiments on the amino acid requirements of chicks by the use of diets in which the protein sources can be considered well characterized.

Glycine.—On the basis of conclusive evidence, glycine has been classified as nonessential, or dispensable, for the rat. A dietary complement of glycine is, however, necessary for the optimum growth of the young chick (7, 8, 9). This requirement amounts to approximately 1 per cent of the diet (7, 8, 10). It is not known whether the chick lacks completely the ability to synthesize glycine or is merely unable to do so at a rate commensurate with the demands for early rapid growth. While other species detoxify benzoic acid by conjuga-

tion with glycine, the fowl couples benzoic acid with ornithine. This peculiarity of the fowl may be related to a difficulty in synthesizing or mobilizing glycine.

Chicks apparently have the ability to synthesize glycine from acetates, which may serve as glycine substitutes in the diet (8). Glycollic acid and betaine are not effective as substitutes for glycine (8). Levels of 2 per cent or more of glycine added to the diet may be toxic to chicks (7) and heavy daily doses of 4 gm. or more have proved fatal to hens (11). Chick embryos with chondrodystrophy contain less glycine than normal embryos (12), probably as a result of poorer formation of cartilaginous and bony tissues, the proteins of which are especially rich in glycine. Some evidence has been obtained (12) for glycine synthesis in the developing chick embryo.

The glycine deficiency syndrome in the chick includes poor growth, muscular underdevelopment, a profound weakness resembling paralysis, and a low muscle creatine content. It is particularly significant that this syndrome is preventable by feeding creatine (8, 10, 13, 14), for which glycine is evidently a precursor in the chick as well as in the rat.¹ The weakness has been identified with a dietary condition formerly thought to be "vitamin B₄" deficiency in the chick (13). Arginine also plays a prominent part in the development and prevention of the syndrome (13).

Coincidental with the poor growth there is an imperfect feather formation (15) which may be alleviated by adding creatine or sources of glycine and arginine to the diet (13, 14). Since chicken feathers contain 6 per cent arginine and 9.5 per cent glycine (16), it has been suggested that the greater effect of glycine and arginine deficiency observed in the case of a rapidly feathering breed as compared to a slowly feathering breed was due to the correspondingly greater demand for these amino acids for feather formation (13). On the other hand, poor feathering may be only a portion of a general condition of inferior development and has been noted in other types of deficiencies, especially that of pantothenic acid.

Young turkeys show no signs of a deficiency of glycine when fed a diet which will produce such a deficiency in chicks (15, 17). This observation indicates that generalities in avian biochemistry may not necessarily be anticipated.

¹ For a discussion of creatine formation in the rat see Schoenheimer, R., and Ratner, S., *Ann. Rev. of Biochem.*, 10, 210-214 (1941); and Beard, H. H., *Ann. Rev. of Biochem.*, 10, 248-253 (1941).

Arginine.—Unlike the rat, the young chick appears to lack any ability to synthesize arginine and rapidly loses weight on a diet very low in arginine (18). The arginine requirement of the young chick is high (19), probably 1 per cent or more of the diet (18). While ornithine is of no value as a substitute for arginine, citrulline is an effective substitute. This fact would indicate that the chick can replace the ureido oxygen of citrulline with an imino group (20).

Since there appears no path from ornithine to citrulline, nor to arginine, it is obvious that the Krebs-Henseleit urea cycle is not completed in the chick. This seems consistent with the fact that the principal end-product of protein metabolism in birds is not urea but uric acid. Chickens on a nonprotein diet or on a diet containing an arginine-free casein hydrolysate excreted less than half the normal amounts of urea. When arginine or whole casein was given to the chickens, the urea excretion rose to a normal value (21). The urea formed by the chick embryo is not derived from the ammonia of general protein catabolism, but may be derived entirely from an active arginine-arginase system present at all stages after the second day of incubation (22). Urea in the embryo does not participate in the formation of uric acid (22). The limited secretion of urea by birds may be accounted for by the action of a small quantity of arginase found principally in the avian kidney. Hence, the detoxification of benzoic acid with ornithine (from arginine) may also be localized in the kidney.

Methionine, cystine, homocystine, and choline.—The essential nature of methionine for the chick has been clearly shown (23). Arachin, the principal protein of the peanut, is deficient in methionine, although well supplied with cystine. As the supplementary protein in chick diets, arachin will not support growth unless methionine is added. It appeared necessary to add 1 per cent of *dl*-methionine to a chick diet containing 20 per cent arachin, 5 per cent gelatin, and 5 per cent dried yeast to achieve an optimum rate of growth. On the other hand, cystine additions to the arachin diet did not promote growth (23).

Proteins of the raw soybean are not well utilized by rats or chicks. The value of the soybean protein is greatly enhanced by heat treatment. While additions of cystine to the raw soybean will correct some of the protein defect, the addition of methionine is more effective (24). It has been suggested that cystine in raw soybean protein is poorly available but that the soybean contains a positive, though slightly sub-optimal, quantity of available methionine. Heat treatment makes the

cystine more available (24). These results with the chick are in agreement with the conception that methionine may serve the purposes of both methionine and cystine, while cystine can meet only the requirements for cystine.

Homocystine will replace methionine efficiently in the chick if ample choline is present in the diet (23). In respect to the necessity of choline for the utilization of homocystine, the chick is completely analogous to the rat.

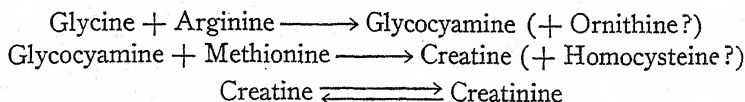
While it has been amply demonstrated that methionine may serve as a precursor of choline in the rat, no such relation is evident in respect to the perosis-preventing and growth-promoting effects of choline in the case of chicks and turkeys. This may be a further point of difference between avian species and the rat (14, 25).

It has been shown that rats convert betaine into glycine by demethylation (26). Furthermore, rats can utilize betaine as a substitute for choline both as a source of methyl groups in the formation of methionine from homocysteine (27) and for other purposes (28). The chick, in contrast, appears unable to utilize betaine to an appreciable extent as a precursor of glycine (8) or of choline (17, 29).

Creatine formation in the chick.—Three amino acids, glycine, arginine, and methionine, are involved in the biological synthesis of creatine in the rat.¹ In the chick, the role of glycine and arginine in this synthesis has been demonstrated (8, 9, 10, 13) in terms of growth, tissue creatine content, and prevention of muscular weakness. It seems probable that methionine is involved in the formation of creatine in the chick, as it is in the rat, but such action remains to be proved. In fact, a methionine-deficient diet which will not support growth does not appreciably inhibit the formation of muscle creatine in the chick (10). Creatine, while exerting a demonstrable growth-promoting action in glycine and arginine deficiency, seems to have no comparable effect in the case of methionine deficiency in chicks (23). Methionine which has contributed methyl groups in the synthesis of creatine may, conceivably, be rapidly re-methylated by choline, hence only small amounts of methionine from the diet or from the tissues may suffice for normal creatine synthesis *in vivo*.

In the following scheme, the biological formation of creatine in the chick has been indicated, insofar as present evidence permits. For example, the improved growth and muscle creatine content on feeding glycocyamine (10) is undoubtedly due to the position of this compound as an intermediate stage in creatine formation in the chick, as

well as in the rat, and to a sparing action of glycocyamine on the immediate precursors, glycine and arginine. The similar favorable effects that are observed on feeding creatinine (10) are most simply explained by assuming that the reaction which forms creatinine from creatine is appreciably reversible in the chick. It should be noted that glycocyamine and creatinine are not as effective as creatine in promoting growth, although all three restore muscle creatine content to the normal range (10).



If ornithine is a by-product of the first step, it is evident that the arginine consumed cannot be regenerated in the chick (20). Assuming that homocysteine is a by-product of the second step, the regeneration of the methionine by reaction of homocysteine with choline (23) may be considered possible in the chick.

It is apparent from the preceding discussion that marked differences exist between the chick and the rat with respect to the metabolism of creatine, choline, and related compounds. The differences may be summarized as follows: (a) The chick, unlike the rat, is largely dependent on dietary sources of glycine and of arginine for the synthesis of creatine. As a result, the chick is subject to a creatine-deficiency syndrome of dietary origin. Such a syndrome has not been described for the rat. (b) Creatinine can apparently serve as a precursor of creatine for chicks but not for rats. (c) Chicks and turkeys, in contrast to rats, appear unable to utilize methionine as a substitute for choline, nor are they able to utilize betaine as a precursor of choline or glycine.

Tryptophane.—The requirement of the young chick for tryptophane, as determined with a diet in which the sole sources of protein were gelatin and acid-hydrolyzed casein, is approximately 0.5 per cent of the diet (30). The optimum growth rate was not quite so high as that obtained when the hydrolyzed casein plus tryptophane was replaced with whole casein. This has been a repeated result in amino acid studies with chicks, the most complete hydrolyzates employed, even those prepared by the use of enzymes, having failed to support quite as rapid growth as the intact proteins (18, 31). The explanation for this finding is not apparent. In view of the inferior results with hydrolyzates, it would appear advisable to conduct investigations

of the amino acid requirements of chicks as far as possible by the use of intact proteins.

Relation of the diet to the amino acid composition of the egg.—The amino acid composition of the proteins of the hen's egg is not noticeably altered by qualitative and quantitative changes in the protein intake of the hen, although rate of egg production may be drastically affected. Analyses were made specifically for tyrosine, tryptophane, and cystine (32), tyrosine, tryptophane, cystine, arginine, histidine, and lysine (33), arginine, cystine, histidine, lysine, and glycine (12).

Uric acid synthesis in the fowl.—Uric acid formation in the pigeon occurs via a purine intermediate, formed in the liver from ammonia or amino acids and a precursor substance, and converted to uric acid in the kidney (34). Pigeon liver slices are able to synthesize xanthine from alanine plus some unidentified carbon source (35). Urea is not a precursor for uric acid in the chick embryo (22) nor of purines in the pigeon liver (34). The surviving liver of the chicken is capable of completing the entire synthesis of uric acid from amino acids, while both liver and kidney are necessary for this process in the pigeon. Urea, with or without tartaric, malonic, mesoxalic, or lactic acid, had no influence on the formation of uric acid (36). The type of purine synthesized by avian liver is yet uncertain, since evidence has been obtained that hypoxanthine rather than xanthine is the primary precursor of uric acid in the pigeon, chicken, and duck (37).

PEROSIS

Perosis is a nutritional disease of young birds, and it has not been recorded as occurring in other vertebrates. The severity of the disease is influenced by heredity (38) or by certain environmental conditions such as crowding and confinement on wire floors without roosts. Perosis is characterized by a shortening and thickening of the bones and is often accompanied by the well-known deformity termed "slipped tendon." The essential nature of manganese in the prevention of perosis was thoroughly reviewed by the discoverers (39). Recently the aggravating effect of certain dietary mineral supplements on perosis has received further attention (40, 41, 42), and it was found that some minerals such as calcium phosphate and ferric hydroxide which are insoluble at the pH of the intestinal tract tend to remove manganese from solution, probably by adsorption (42).

A dietary factor of organic nature is also necessary for the preven-

tion of perosis. The existence of a thermolabile antiperotic factor in cereal grains had been known for some time (43). It was shown that, under certain conditions, manganese tended to aggravate perosis in turkeys while a strain of yeast partially alleviated the condition (44). An organic antiperotic factor was extracted from liver by alcohol (45). Choline was found to prevent perosis (46, 17). Perosis did not appear if the basal low-choline diet was also deficient in the precursors of creatine (15). It was suggested that the tension exerted on the bones by the muscles played a part in causing perosis; hence, when the tension was reduced by creatine deficiency, which causes a muscular weakness, the tendency to distortion of the bones was lessened (15). Mechanical tension has been shown to increase the formation of cartilage in chick bones (47). Other reports (48, 49) indicated that certain yeasts supplied an antiperotic factor other than manganese and that choline reduced or prevented perosis (50, 51). A recent report (52) indicates the existence of an antiperotic factor which was differentiated from choline and manganese. The factor was present in a water extract of liver and was adsorbed on fullers' earth, from which it was eluted by ammonia. This factor appeared to be needed in addition to choline, while the factor in yeast (49) was stated to be able to replace choline in the prevention of perosis. Undoubtedly the choline present in natural foods contributes to their activity in preventing perosis, but the presence of other antiperotic factors is being actively investigated.

The egg white syndrome in chicks was observed to be accompanied by perosis (53), which may indicate that biotin prevents perosis.

Other functions of choline.—In addition to preventing perosis, choline stimulates growth in turkeys and chicks (46, 47, 51). Studies of certain choline analogues (29, 54) indicate that the antiperotic and growth-promoting properties of choline may be chemically differentiated. Arsenocholine prevents perosis (17) and has been shown in studies with rats (56) not to furnish labile methyl groups. Hence, the possibility is indicated that the antiperotic function of choline may be unrelated to its "methylating" activity.

It was reported (55) that egg production was increased by feeding choline to birds fed a basal diet consisting principally of polished rice and dried skim milk, supplemented with vitamin concentrates and minerals. Choline was also reported to decrease mortality and to prevent the abortion of egg yolks. The authors state that choline decreased

the fatty acid content of the livers of laying birds; however, the values for fatty acids reported all fall well within the range reported elsewhere (57, 58) for laying pullets on a normal diet.

Phosphorus metabolism and perosis.—A diminished phosphatase activity was noted (59) in the blood and bones of perotic chicks on a diet deficient in manganese. The bones of perotic chicks on a diet deficient in choline, however, were normal with respect to phosphatase activity (51). A slight decrease in the calcium and an increase in the inorganic phosphorus content of the blood serum were reported in perotic turkeys (60).

VITAMIN STUDIES

Fat-soluble vitamins.—Work with avian species on vitamins A, D, E, and K has recently been reviewed (1, 4, 6, 61, 62). Three carotenoids were separated from the retinas of chickens (63). These possessed the properties of xanthophyll, astacene, and an unidentified hydrocarbon. The latter two compounds may be synthesized during embryonic development, and the authors discuss the relation of their findings to color vision in the chicken. Vitamin A was found in the intestinal tissue of certain sea-birds (64). The precursors of vitamin D in the external coverings of chickens were found to be present in the largest concentration in the skin of the legs and feet (65). There was some indication of the presence of 7-dehydrocholesterol and ergosterol in an extract of leg skin. Recent work on the biological assay of vitamin D with chicks, a problem of much economic importance, was discussed (66).

The addition of elemental sulfur to the diet produced symptoms of vitamin D deficiency in chickens that were dependent on fish oil as a source of vitamin D (67, 68). The symptoms were counteracted by feeding a high level of fish oil or by exposing the birds to sunlight.

It was found (69) that (*dl*) α -tocopherol was more than thirty times as active as (*dl*) β -tocopherol in preventing alimentary exudative diathesis in chicks.

Thiamin, riboflavin, and pyridoxin.—Experimental work with these vitamins was reviewed in the chapters on "Water-Soluble Vitamins" in previous issues of the *Annual Review of Biochemistry*. A comprehensive account of riboflavin in poultry nutrition appeared recently (70). Chicks require between three and five parts of pyridoxin per million parts of diet (71).

Nicotinic acid.—A dietary deficiency of nicotinic acid has not been

demonstrated in birds, and it has recently been shown that this substance is synthesized by the chick during embryonic development (72, 73), so that the newly hatched chick contained from ten to twenty times as much nicotinic acid as the unincubated egg.

Pantothenic acid.—It was found (74) that the pantothenic acid content of hens' eggs was proportional to the diet, and that the content increased rapidly when pantothenic acid was added to the diet. The injection of pantothenic acid into eggs was found to change the subsequent development of chick embryos (75, 76). The changes were observed in the size of certain organs and in the blood hemoglobin concentration. Changes were also caused (75) by injecting thiamin, riboflavin, or nicotinic acid. Addition of pantothenic acid to the diet of the parent hens (75, 76) affected the embryos, causing an increase in blood hemoglobin, changes in the sizes of the brain and heart, and changes in the B-vitamin content of the liver, brain, and heart. The viability of the embryos was improved. It was reported (77) that pantothenic acid was necessary for reproduction in hens.

Biotin.—Like other animals, chicks (78) are subject to a characteristic syndrome when fed a diet containing uncooked egg white. Egg white contains a small amount of a specific protein, "avidin," which combines with biotin. Avidin and its relation to the egg white syndrome are discussed elsewhere in this volume. The syndrome in chicks is marked by keratinization and fissuring of the skin of the feet, by other symptoms of dermatitis, and by a lowering of the biotin content of the tissues (79). In rats, the egg white syndrome is cured by biotin (80).

Symptoms similar to those of the egg white syndrome were reported as occurring in chicks receiving certain simplified diets (81) or a diet consisting of heated cereals supplemented with purified casein, synthetic B-vitamins, minerals, and cod liver oil (82). In both cases (81, 83) the symptoms were alleviated by biotin concentrates, and in the latter case (83) it was reported that three doses of 10 μ g. of crystalline biotin methyl ester completely cured the syndrome. A concentrate fed daily at a level supplying only 1.25 μ g. of biotin was also effective.

The characteristic involvement of the feet which occurred in chicks which received a certain heated diet (82, 83) was not reported in chicks receiving the heated diet used for pantothenic acid assay (84). The difference may have been due in the latter case to the use of whey adsorbate rather than purified vitamins, to other differences in the

basal diet, or to the use of a preliminary feeding period on a normal diet (84).

An acute form of dermatitis occurring in turkey poultts was prevented by a crude concentrate containing biotin (85). Similar symptoms have been observed in the writers' laboratory in poultts receiving a diet containing unheated egg white. The etiology and appearance of the condition appear to differ from the dermatitis which is prevented by riboflavin (86).

Inositol.—This substance is present as phytic acid in avian erythrocytes in comparatively large amounts (87). The inositol content of newly-hatched chicks was found to be six times as great as in unin-cubated eggs (73). For many years there had existed evidence which suggested synthesis of inositol by the chick embryo (88). However, it was reported that growth of chicks was increased somewhat when inositol was added to certain special diets containing "kidney residue" or blackstrap molasses (89).

Norite eluate factor ("Folic acid").—Preliminary results indicate that crude sources of this new member of the vitamin B complex increase the growth of chicks on a purified diet (90). The factor is distributed throughout the tissues of chicks (91) and is especially concentrated in the liver. Further reports on the biochemistry of the factor will be awaited with interest.

Factors "R," "S," and "U."—These terms have been applied to various fractions which have been prepared from yeast and which promote the growth of chicks on simplified or special diets. Improvements in egg production and hatchability have also been reported (92). The relation of these factors to the newer members of the vitamin B complex, including biotin and folic acid, awaits clarification. The addition of choline to a ration deficient in factors R and S was reported (93) to reduce the incidence of perosis and to increase growth markedly.

MISCELLANEOUS NUTRITIONAL STUDIES

Nutritional anemia.—An anemia characterized by abnormal erythrocytes was produced in pigeons on a purified diet (94). A dietary dermatitis of pigeons was accompanied by anemia (95). Chicks on a simplified diet developed a macrocytic anemia (96). The deficiency was not ascribable to a lack of any of the known vitamins.

Rice factor.—A factor present in polished rice and other cereal products but absent in appreciable quantities in dried yeast is re-

quired in the diet of young chicks for optimum rates of growth (97, 98, 99). Presence of the factor in kidney and liver has also been reported (100). This factor was found to be dual in nature; one component was identified as glycine, and the other was found present in chondroitin (7). Chondroitin had been previously reported to exert a growth-promoting effect in the diet of the chick (101). The component of the chondroitin molecule specifically responsible for this activity was identified as glucuronic acid (102). Subsequently, other carbohydrates and carbohydrate derivatives were found to possess similar activity, thus showing that no one specific compound was required (103). The most effective compounds were *d*-glucuronic acid and *d*-arabinose. The more commonly occurring *l*-arabinose appeared less potent than *d*-arabinose. Less growth-promoting also were *d*-xylose, *d*-ribose, rhamnose, galactonic lactone, and calcium gluconate. Natural sources of glucuronic acid and pentoses (gum arabic), of mannuronic acid (sodium alginate), but not of galacturonic acid (gum tragacanth and pectin), manifested potency (103). Reasons for the necessity of such substances in the chick's diet are as yet unknown. The growth-promoting activities of chondroitin, arabinose, and xylose (9) and of gum arabic (14, 15) have been confirmed.

Nutritional gizzard lesions.—A nutritional disorder of the chick gizzard characterized by localized erosion or necrosis of the secreted lining and sometimes involving the epithelium has been noted in a number of investigations employing simplified experimental chick diets, especially those low in fat or free of green plant tissue. The early work on this subject has been adequately reviewed (104, 105, 106).

It is generally agreed that the crater-like lesions of the gizzard lining are the result of a dietary deficiency, but very contradictory reports on the properties of the preventive factor have been issued, including the suggestion that it might be identical with the hypothetical "vitamin B₈" (107) and, on the other hand, that the factor is fat-soluble and distinct from any of the other fat-soluble vitamins (104, 108, 109).

Chondroitin has been reported to prevent gizzard erosions occurring in chicks on certain experimental diets (110), but in other investigations this substance was not found to be appreciably active (111, 101, 112).

A clue to the probable nature of the "gizzard factor" was provided in the finding that bile and bile acids in the diet of the chick will prevent lesions of the gizzard lining very effectively (113). Cholic acid

was the most active of the natural bile acids tested, while dehydrocholic acid, an artificial product, was almost equally active (114). The activity of cholic acid in the prevention and cure of nutritional gizzard lesions of the chick has been confirmed (112, 9). In marked contrast to beef bile, hog bile is comparatively ineffective against gizzard lesions of the chick (112). Hog bile contains very little cholic acid.

Cholic acid was determined in the chick gall bladder bile and was also found in combination with the gizzard lining, to a slight extent in cases of severe erosion and to a greater extent when cholic acid was fed. It was suggested that any condition which might inhibit the normal secretion of bile, directly or indirectly, might lead to gizzard erosion (114).

Since practical chick diets which are not conducive to gizzard erosion do not contain appreciable amounts of cholic or other bile acids, it seems probable that the source of the erosion-preventing action in such diets may be some precursor required by the chick for the normal secretion of bile acids (114). Cholesterol and other sterols do not appear to manifest such precursor properties (112). Little progress has been made in the identification of a bile acid precursor in the chick, but further evidence for the existence of such a substance has been obtained. Whole milk and skim milk, given in place of drinking water, reduce the incidence and severity of gizzard erosions and increase the amount of cholic acid present in the gall bladder bile of the chick. The active factor remains in liquid milk after boiling and distillation, but is absent from commercially-prepared dried-milk products, which have no similar effect either on erosions or on cholic acid formation in the chick (115).

Cinchophen added to a normal diet for the chick will produce lesions of the gizzard lining which resemble closely those developed in chicks on a gizzard factor-deficient diet (112, 116). It has long been known that cinchophen administered orally or intravenously may cause gastric ulcers in mammals. Ox bile salts, cholic acid (112), and liquid milk products (115, 116) counteract the cinchophen-induced gizzard lesions, as well as the deficiency lesions. The role of bile in the maintenance of the gizzard lining seems clearly established by these findings.

The formation of gizzard lesion has been stated to originate as small hemorrhages in the capillaries of the submucosa (117). Such hemorrhages were apparently coincident with a failure of bile passage from the gall bladder into the duodenum, as indicated by absence of

bile in the cystic duct (118). Ligation of the bile duct of adult chickens produced a hemorrhagic condition in the gizzard. It was suggested that biliary dysfunction might lead to poor absorption of vitamin K and a consequent hemorrhagic tendency in the gizzard wall (118). On the other hand, evidence has been presented that vitamin K is not a primary factor in this disorder (104, 108, 112). The fact that cholic acid is much more active against erosions than deoxycholic acid, while the latter is known to be particularly effective in promoting absorption of the fat-soluble vitamins, does not appear to favor the explanation that the role of bile and of cholic acid is merely that of a carrier of an antierosion factor (108).

MINERAL METABOLISM

This topic is the subject of recent reviews (2, 3).

Calcium and phosphorus.—After injection of radioactive phosphorus a greater concentration of radioactive material was found in the bones of rachitic chicks than in the bones of normal chicks (119). Phytic acid was not well utilized as a source of phosphorus by chickens (120, 121). No effect on ossification in chicks was produced by adding various sodium, potassium, and ammonium salts to the diet (121). Increases in plasma phosphorus and phosphatase were observed during shell deposition in hens (122). Shell deposition is also accompanied by a mobilization of skeletal calcium, and the fraction of the bone minerals that is mobilized was stated to have a higher calcium/phosphorus ratio than that of the skeleton as a whole (123). The egg-laying cycle in pigeons was accompanied by histological changes in the bones (124). Deposition of calcium in the egg shell of the chicken is so rapid that the calcium requirement is seldom supplied by the food ingested during this period and the skeletal calcium in used as a reservoir (125). It is suggested (126) that carbonic anhydrase may be concerned in shell formation.

Potassium.—Chicks were found to need the high level of at least 0.172 per cent of potassium in the diet for maximum growth and 0.13 per cent to prevent heavy mortality (127).

Manganese.—Micromelia (128) has been added to the list of effects (39) of manganese deficiency in chickens, which also includes perosis, low egg production, reduced ash content of egg shells, ataxia, low hatchability, deformed embryos, and other symptoms. Manganese was found to be absorbed at a slow but definite rate from an intestinal loop containing a solution of manganese sulphate (129).

Iodine.—Recent studies (130) place the iodine requirement of chickens higher than values estimated previously. At least 1 p.p.m. of iodine in the diet was required for hatchability in chickens. Feather growth of young chickens was improved by feeding iodinated casein.

HORMONES

Striking and varied responses may be produced by the administration of hormones to birds. The effects produced by estrogens and androgens have been of particular interest to investigators, and much activity continues in this field. A description of the numerous characteristics of birds which are influenced by the sex hormones appears in a recent review (131). Many investigations of the relation of endocrine factors to avian biochemistry have been reviewed in the chapters on "Hormones" in preceding issues of the *Annual Review of Biochemistry* [see also (132)].

Estrogens.—The onset of egg-laying is accompanied by marked biochemical changes, many of which are duplicated after the administration of estrogens. Estrogens have been shown to cause pronounced increases in the blood lipids of male or immature female chicks (133, 134, 135) and pigeons (136). Stilbestrol was more effective than estradiol benzoate (133). Both these substances were more effective on blood lipids than estrone (133, 134), while estrone was more effective than estradiol (133) on the basis of rat units. Estrogens also produced an increase in the serum calcium of male and female pigeons and other birds (132). Again, estrone or estradiol benzoate was more effective than estradiol (137). Estrin also produced a marked increase in the size of the oviduct (138) and caused a spreading of the pubic bones (139).

It has also been noted (140) that estradiol produced increases in ossification of the long bones and in blood phosphatase. The hyperossification was more marked in Pekin ducks than in Mallard ducks, and the authors draw attention to the fact that the former breed are inherently more productive of eggs than are Mallards.

Estrogens cause certain effects that are not associated with egg production. These effects include various changes in the morphology and coloring of the feathers (131, 141), a reduction in the size of the bursa of Fabricius, and in the length of the tibiae (139). Estrone causes a reduction in the size of the comb of male chickens (142) and a partial sex reversal in male embryos of birds (139).

Androgens.—The most conspicuous action of estrogens on birds of both sexes is the activation of certain processes which are associated

with the production of eggs. In contrast, the outstanding effect of androgens is upon behavior. Testosterone induces crowing and aggressiveness in chicks (143), social aggressiveness in hens (144), and singing and other manifestations of male behavior in female canaries (145). In certain species, such as the black-crowned night heron, testosterone produces the normal secondary sexual characteristics in both sexes, together with sexual behavior (146). Androgens enhance many of the secondary characters associated with the male sex in birds, including growth of the comb and wattles in chickens of both sexes and growth of the testes of male chickens. The earlier work has been reviewed (131, 147).

The stimulating action of androgens on the growth of combs of chickens is inhibited by estrogens (148), but these hormones exert a synergistic action in the starling, in which species the androgens promote growth of the oviduct (149).

Progesterone.—No effects have been produced by the injection of progesterone into birds.

Gonadotropic hormones.—These hormones cause an increase in the size and activity of the gonads, resulting in the appearance of the effects known to be produced by estrogens and androgens. Ovulation has not been produced in birds by administration of gonadotropic preparations. Other changes, coincident with egg production and caused by gonadotropic hormone, but not yet examined with respect to estrogens, include a rise in the various phosphorus fractions of the plasma, including the serum vitellin (150).

Adrenal cortical hormones.—The effects of adrenalectomy in sexually developed male chickens (151) resulted in death in from six to fifteen hours unless cortical hormone and sodium chloride were administered, in which case the birds survived for many days. Similar results were obtained with drakes (152), and the amount of desoxycorticosterone required for survival was proportional to the size of the testes. Desoxycorticosterone was without effect on comb growth (153) or on blood lipids (154).

Thyroid.—Egg production by chickens was reduced by thyroidectomy (155, 156), and the administration of thyroxine to the thyroidectomized chickens resulted in an increase in egg production (156).

Insulin.—Depancreatized chickens and ducks are able to utilize carbohydrate normally without receiving insulin. In ducks (157), in contrast to dogs, a greater degree of ketonemia developed after fasting in normal individuals than in depancreatized individuals.

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ANIMAL PIGMENTS

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Four years have elapsed since the last review of this subject by Lemberg (157). The obligation of comprehensive coverage of the literature accumulated in the field during this period cannot be met. Related reviews, however, have dealt with certain aspects of the animal pigments (158). Need for conservation of space has dictated the omission of certain pigments—the flavins and visual purple—treated elsewhere^{1,2} in the present volume.

During the past four years significant progress has been made through well-planned, comprehensive programs of research in the field of metalloporphyrins and their derivatives. Such contributions have come mainly from the laboratories of Clark, Lemberg, Theorell, and Pauling. The allocation of space in this review therefore will favor the porphyrins and the iron porphyrin pigment derivatives. The work of Clark and colleagues (1 to 5) already has been reviewed from the standpoint of oxidation-reduction (158d).

Physical methods of measurement continue to dominate in this field. Of special interest is the determination of magnetic susceptibility, which has yielded results of evident importance. Since this analytical procedure is novel in its biological applications, the reviewer has undertaken to describe it and its background in some detail.

PORPHYRINS

Porphyrins of natural hemin.—It has been assumed that the porphyrins of the respiratory pigments (hemoglobin, myoglobin, cytochrome-c, catalase, etc.) are solely of etioporphyrin type III,³ the most common representative of which is protoporphyrin IX (Figure 1a).

¹ Cf. p. 1.

² Cf. p. 465.

³ There are four structural types of porphyrins, which correspond to four synthetic etioporphyrin types. In Fischer's system of notation (7) Roman numerals designate a particular isomer and not the structural type. Thus protoporphyrin IX, mesoporphyrin IX, and coproporphyrin III belong to the same structural type III, which is determined by a characteristic arrangement of substituent groups (Figure 1a).

Fischer (6) in preparing mesoporphyrin from the protoporphyrin of natural hemin (derived from hemoglobin) has isolated two mesoporphyrins, IX and II. Mesoporphyrin II belongs to etioporphyrin type I (Figure 1a). Each structural type of porphyrin is considered to have an independent synthetic origin from simple pyrroles, since the change from one type into another by a shift in the positions of the substituted groups has not been possible in the laboratory (7). It is most probable, therefore, that mesoporphyrin II originated from a precursor other than protoporphyrin IX. This discovery suggests that porphyrins of both types III and I may have to be considered in the configurations of the respiratory pigments. Fischer's finding also lends support to his theory of the independent biological syntheses ("dualism") of the above types of porphyrins (Figure 1b).

Origin and metabolism.—The hypothesis of the dual origin of porphyrins type III and I has been further developed and applied by Rimington (8), Dobriner (9), Vigliani (10), and their colleagues to explain the abnormal increase in excretion of porphyrins type I (coproporphyrin I and uroporphyrin I) in congenital porphyria, an inheritable anomaly of metabolism in humans and bovine species, and in other abnormal conditions such as hemorrhage (11), pernicious anemia (9), and pellagra (12). These investigators postulate that normally both types of porphyrin are produced independently in the organism, the biological synthesis of type III being predominant. Under the above conditions, associated with increased hemopoiesis, the normal ratio of production of porphyrin types is disturbed in favor of type I. Enzymic control (8) may determine the ratio of synthesis of types III and I. On the other hand, in intoxications with lead (13) and sulfonamides (14) and in aplastic anemia (15) increased porphyrin excretion occurs, but type III porphyrins predominate as in the normal organism. In intoxications overproduction of porphyrins may be associated with a deterioration of liver function (16).

The scheme of metabolic pathways, shown in Figure 1b, is that of Dobriner & Rhoads (17), modified to include only known structures and to present certain other points of interest. The pyrromethenes of the type shown have been synthesized and have been derived from the bile pigments (7). It is assumed that such pyrromethenes are produced from simpler pyrroles and then condense together, as schematically presented, to produce porphyrins type III and I. The simpler building stones are not known, nor is it known why normally union of A and B predominates over that of A and A, nor why union of B

and B presumably does not occur *in vivo*. Union of B and B would produce porphyrins of type II, which have not been isolated from natural products.

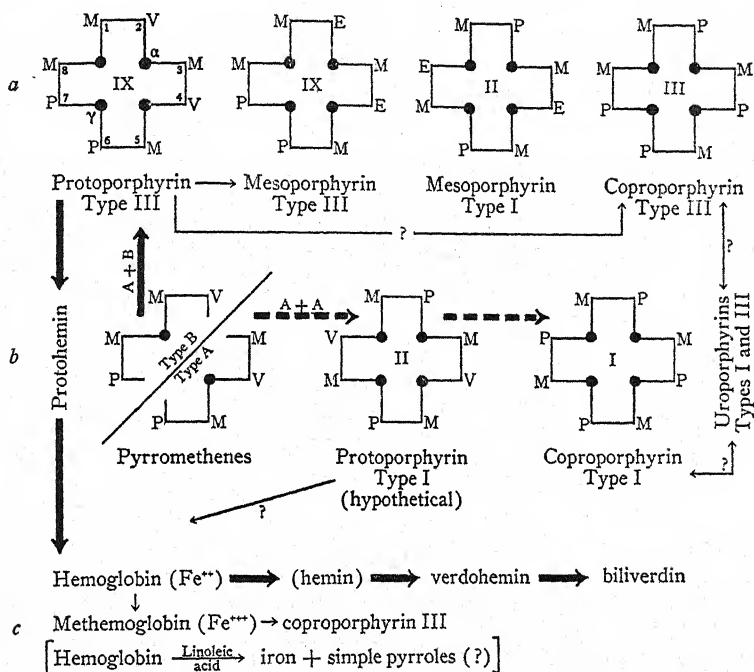


FIG. 1.—Symbolic structural representations of various porphyrins isolated from natural sources and pathways of their metabolism. Heavy arrows denote more prominent pathways. Roman and Arabic numerals in *a* and *b* follow Fischer's notation (7). Letters M, V, and P stand respectively for methyl, vinyl, and propionic groups. Solid circles represent methene bridges between two pyrroles. Union of pyrromethenes A and B by a methene bridge in the γ -position would symbolize the structure of biliverdin, and this structure with central iron would represent verdohemin.

Lemberg (18, 157) has stressed the view that the normal catabolic path of hemoglobin (Fe²⁺) leads to bile pigments (Figure 1c) and not to porphyrins.⁴ Of interest in this connection is the finding that coproporphyrin III is excreted in abnormal quantities in rats poisoned with derivatives of aniline (19) and with various sulfonamides (20). Both

⁴ Cf. p. 536.

classes of drugs also produce methemoglobinemia. It is deduced, therefore, (19, 20) that methemoglobin (Fe^{+++}) is at least partly catabolized to porphyrins type III (Figure 1c). Oxidation of the iron may be responsible for the altered metabolic path of methemoglobin (19).

Progress in the isolation of naturally occurring porphyrins has depended upon the development of suitable quantitative methods for the separation of mixtures. Zeile & Rau (21) have improved an earlier ether-hydrochloric acid partition procedure. Optimal conditions for the separation of various porphyrins by their partition in the acid and ether layers have been established. The method has led to the first demonstration of the occurrence of mesoporphyrin in biological material (feces). The excretion of urinary porphyrins (without distinguishing their types) has been measured quantitatively in four normal human subjects by Tropp & Siegler (22). Upon a vegetable diet 18 to 100 μg . per day were excreted, 70 to 85 per cent being eliminated during the period of active metabolism. The addition of meat proteins to the diet produced an appreciable increase in porphyrin excretion; addition of milk proteins had no effect. The conclusion was reached that the metabolism of porphyrins is largely endogenous. It is of interest to note that a case of fatal poisoning presumably with illuminating gas (*gasvergiftung*) has been listed among conditions associated with methemoglobinemia and coproporphyrinuria (23).

Porphine, isoporphine, and azaporphines.—The possibility that a special type of isomerism, N-isomerism (I and II, Figure 2), may exist in the porphyrins has been suggested by Corwin & Quattlebaum (24), although earlier work (25) lent little support to the idea. The existence of such isomers now appears to be established by Rothmund's synthesis of isoporphine (26). This new porphyrin was isolated from the low hydrochloric acid fractions in the synthesis of the basic porphyrin unit, porphine, through pyrrole-formaldehyde condensation (27). Iron (hemin), magnesium (phyllin), and copper complexes of both porphine and isoporphine were prepared and compared (26). The spectra of isoporphine, its hemin, and phyllin differ from those of porphine and its derivatives by appreciable displacement towards the longer wave lengths. This finding suggests the possibility that isomerism is not lost upon the introduction of the metal into the porphine ring (cf. 25). From a consideration of a space model of porphine Huggins (24) has suggested that adjacent secondary and tertiary nitrogens might be joined by hydrogen bridges (III, Fig-

ure 2). Isomerism could result from an exchange of partners by the hydrogens. This type of isomerism, involving two $N-H \leftarrow N$ bonds, would be destroyed by the substitution of a functional group for hydrogen and appears to be definitely unlikely from Rothmund's finding. An attempt to demonstrate $N-H \leftarrow N$ bonding in pyromethenes and etioporphyrin I (28) by means of infrared spectrophotometry has not been successful. In this work it was deduced that the vibrations of the $N-H$ groups of the porphyrin were responsible for the absorption band at 3.01μ , since this band disappeared upon conversion of the etioporphyrin into etiohemin. The unbonded $N-H$ group of pyrrole is located at 2.85μ (29). The appreciable difference between these maxima of absorption invites the temptation to conclude that $N-H \leftarrow N$ bonding, rather than $N-H$ groups, may exist in the porphyrins. However, this criterion is not reliable since large variations exist in the location of vibration bands due to the presence of the same group in different compounds (30). The slight distortion from true tetragonal symmetry of the crystal structure of azaporphines (see p. 536) has been taken as evidence for the existence of hydrogen bonds in such molecules (31).

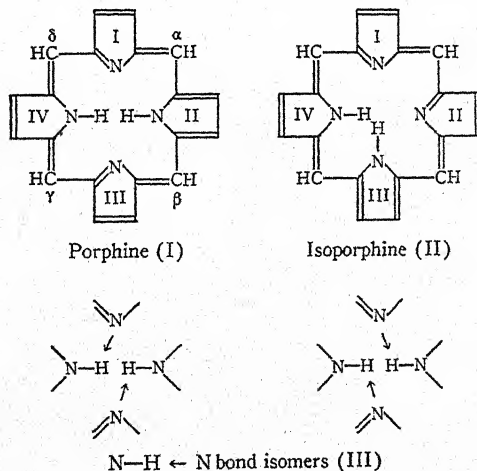


FIG. 2

The syntheses of tetrabenzoisoporphine and tetrabenzoporphine have been accomplished respectively by Helberger & Rebay (32) and by Barrett *et al.* (33). This is another example of N-isomerism. These

derivatives have additional interest since they represent essential compounds in the comparative study of the molecular structures of porphine and the related dyestuff phthalocyanine (tetrabenzotetraazaporphine). Due to their common possession of four isoindole groups tetrabenzoporphine and phthalocyanine are particularly close analogues. As in the case of phthalocyanine and its metallic salts (34), the benzoporphine has yielded to direct x-ray analysis (35). It belongs to the same space group as the azaporphine but differs considerably from the latter in cell dimensions. The assumption that the molecular structure of porphine is planar appears justified, the inner nucleus being a closed system with twelve carbon and four nitrogen atoms. For a discussion of x-ray analysis of the structure of organic molecules the reader is referred to a review by Robertson (36).

The absorption spectra (700 to 360 m μ) of methene-substituted porphines, prepared by Rothmund's synthesis, and their metallic salts have been measured quantitatively by Knorr & Albers (37). Aronoff & Weast (38) have studied the spectra of several porphyrins and their acid salts. Monoacid salts could not be demonstrated spectroscopically. Intermediate types of spectra corresponded to those expected for mixtures of two forms, the free base and the diacid salts (25). The applications of fluorescence spectrophotometry have been reviewed by Dhéré (39).

The penetration of lipid and protein monolayers by porphyrins, protohemin, and bilirubin has been investigated (40). Capillary-active porphyrins (protoporphyrin IX) and bilirubin were found to penetrate such monolayers strongly, penetration being influenced largely by interaction of the hydrophobic (nonpolar) part of the molecule with the film. These findings are thought (40) to throw some light upon the mechanism of formation of porphyrin complexes with globin, studied by Holden (41), and the association complexes of bile pigments with albumin in icteric sera and with high molecular weight substances in the bile (42).

BILE PIGMENTS

Origin: verdohemochromogens and choleglobin.—It is convenient to treat as a unit in this section the contributions upon verdohemochromogens and related substances. The active prosecution of this field began with Lemberg's demonstration (43) that the "green hemin" of Warburg & Negelein (44) is the ferrichloride of biliverdin (verdohemin), formed from protohemin by the oxidative scission of the por-

pyrrole ring in the α -position. Nitrogenous derivatives (pyridine, etc.) of verdohemin after reduction (analogues of corresponding nitrogenous derivatives of ferroprotoporphyrin)⁵ were named "verdohemochromogens" (43). Heubner (45) and Havemann (46) use this term in a generic sense for any derivative which contains the type of open-ring iron porphyrin capable of uniting with native globin to form verdoglobin. Lemberg *et al.* (47, 48) have more recently described two new precursors of bile pigments, differentiating them from the verdo- derivatives by the terms "cholehemochromogen" and "choleglobin." The introduction of additional names resulted from presumptive spectroscopic evidence of possible constitutional differences in the prosthetic groups of the verdo- and chole- classes of compounds. An unsuccessful attempt was made (49) to prove the existence of different structures by a study of the products obtained from the acid splitting of pyridine verdohemochromogen and choleglobin. Very similar products were found in each case. This question must remain an open one.

Verdoglobin and choleglobin are apparently very closely related. Choleglobin may be defined as bile pigment-hemoglobin, a combination of native globin with an open-ring iron porphyrin. Cholehemochromogen, derived from choleglobin through the action of alkali and a reducing agent (47), is an analogue of denatured globin hemochromogen. Barkan & Schales (50) independently have postulated the existence of a bile pigment-hemoglobin, which they designated "pseudohemoglobin." Their green pigment was no doubt the same as Havemann's verdoglobin (46) since similar methods of preparation were employed (see p. 539). According to Lemberg, Legge & Lockwood (48), who have repeated Barkan & Schales' procedure, choleglobin and pseudohemoglobin are not identical substances. The latter is rather a cyanide derivative of ferrous cholehemochromogen.

If one surmounts the perplexities of the present terminology, all the above substances—verdohemochromogens, cholehemoglobin, and pseudohemoglobin—are green pigments whose prosthetic groups are probably rather similar open-ring iron porphyrins. The breach in the tetrapyrrolic shell has somehow rendered the iron labile, so that it is easily split out by acids with the formation of bile pigments—bilverdin (dehydrobilirubin) and biliviolins (49).

Several different methods have been employed for the production

⁵ Cf. p. 542 for nomenclature of metalloporphyrin derivatives.

in vitro of green hemin derivatives: (i) Lemberg's procedure (18, 47) involves a coupled aerobic oxidation of ascorbic acid and either pyridine ferroprotoporphyrin or hemoglobin. In the case of buffered solutions (pH 7.2 or 8.5) of oxyhemoglobin mixed with ascorbic acid and exposed to air the reaction proceeds rapidly at 37° C. The typical absorption band in the red region (maximum at 630 mμ) of reduced choleglobin quickly appears. The reaction is a complicated one. In the early stages (first 45 min.) mixtures of oxyhemoglobin, hemoglobin, choleglobin, and probably some methemoglobin are present. Although choleglobin cannot be separated from the other components (47, 48), the early reaction may be followed quantitatively. The increase in absorption at 630 mμ parallels the increase in labile or easily split iron (51). After conversion of the pigments in the reaction mixture into their corresponding carbon monoxide hemochromogens by means of carbon monoxide, sodium hydroxide, and dithionite (hydro-sulfite), they respond to spectrophotometric treatment as a two-component mixture (52). It is recognized that the spectra of carbon monoxide hemochromogens and carbon monoxide hemoglobin are very similar (53). The spectra of carbonyl cholehemochromogens and carbonyl choleglobin are also similar to each other, but they differ spectroscopically from the carbonyl hemochromogens (48). The latter derivatives have spectra with two bands in the green region; the former, a prominent band in the red region. This difference is at the basis of the method of analysis used by Lemberg *et al.* It is of interest that while choleglobin unites with carbon monoxide, no prominent spectroscopic change accompanies this reaction. The band remains at 630 mμ (48). Satisfactory evidence for the postulated reversible union of choleglobin with oxygen (48) has not been furnished. By means of the above spectrophotometric procedure the reaction velocity of choleglobin formation is found to be dependent upon pH and oxygen pressure (52). The temperature coefficient of the reaction is high, and the molar ratio of ascorbic acid oxidized to choleglobin formed is of the order of ten to one. At later stages (one to two hours) in the coupled oxidation of ascorbic acid and hemoglobin the situation becomes even more complicated through secondary changes in the pigment products (48, 52).

(ii) Another method for producing choleglobin is by means of low concentrations of hydrogen peroxide, with or without ascorbic acid (52). That choleglobin can be produced by such apparently different agents as peroxide and ascorbic acid is explainable by the postu-

lated mechanism of the reaction (18, 52). The hydrogen yielded by the formation of dehydroascorbic acid is transferred to oxyhemoglobin with the formation of a theoretical ferrous hemoglobin-peroxide complex. The bound peroxide undergoes an intramolecular peroxidative reaction resulting in the irreversible oxidation of the porphyrin ring. It is probable that ring rupture is a secondary reaction, derivatives of ferric hydroxyporphyrins (hydroxyl group on the α -methene carbon) being first produced (18, 47). It is not clear, however, whether such intermediates are already green pigments (see p. 540), or whether ring rupture is required for the color change.

Pseudohemoglobin (50) and Havemann's verdoglobin (46) are both produced from hemoglobin by means of peroxide in the presence of cyanide. Havemann has submitted evidence that three molecules of peroxide per atom of hemoglobin iron are required in the formation of verdoglobin. The reaction may be written as follows: hemoglobin + 3 peroxide \rightarrow verdohemoglobin + carbon dioxide + 2 water. This is consonant with Lemberg's formulation of the structure of the open-ring iron porphyrins. It has already been mentioned that Lemberg and colleagues (48) appraise the effect of cyanide as merely a complicating factor in the reaction. On the other hand, Havemann (46) and Jung (54) believe that all agents (cyanide included) which inhibit the action of catalase and oxidation enzymes promote the formation of bile pigment precursors.

(iii) Barkan & Schales (55) have postulated an open-ring structure for the prosthetic group of sulfhemoglobin. This conclusion has been accepted by others (46, 54), although it is founded upon rather meagre presumptive evidence, namely, the green color of hemoglobin solutions treated with hydrogen sulfide, the absorption band in the red spectral region, and the increase in easily split iron in such solutions. The mechanism of formation of sulfhemoglobin from oxyhemoglobin by means of sulfide resembles that of the formation of bile pigment-hemoglobin. Hydrogen sulfide is effective only in the presence of oxygen—a phenomenon explained by the probable formation of peroxide (55, 56), a necessary component in the reaction.

Lemberg and co-workers (48) do not support the idea that sulfhemoglobin belongs to the class of open-ring iron porphyrin derivatives. Nor is Barkan & Schales's conclusion supported by spectroscopic evidence of hemochromogen formation from sulfhemoglobin-hemoglobin mixtures. Upon treatment with alkali or pyridine such solutions yield typical spectra of reduced globin hemochromogen

or pyridine ferroprotoporphyrin (57, 58) rather than chole- or verdohemochromogens with distinct bands in the red. According to Venndt (59) the measurement of iron split from hemolyzed blood solutions by means of dilute acids is not a reliable criterion of the presence of verdohemochromogens in blood. The amount of iron split was found to be a function of the acid (acetic being more effective than hydrochloric), its concentration (0.1 per cent hydrochloric being more effective than 0.4 per cent), time, and temperature. Recently Haurowitz (60) published the interesting observation that neither sulfhemoglobin nor sulfheminproteose (produced from the former by peptic digestion) lose iron on treatment with dilute acids; hence they cannot be open-ring structures. Hydrolysis of sulfheminproteose by means of boiling hydrochloric acid resulted in a porphyrin, which still contained sulfur. The analyses indicate that this new porphyrin differs from protoporphyrin by an excess of two sulfur and four oxygen atoms, leading Haurowitz to postulate two sulfur dioxide bridges between methene carbons and side chains. It is evident that the structure of the prosthetic group of sulfhemoglobin requires further study.

(iv) Synthetic work in Fischer's laboratory has suggested that green pigments may be produced without an opening of the porphyrin ring, and that these substances may serve as precursors of bile pigments. The question is perhaps somewhat academic since on its answer depends merely the stage at which ring scission occurs, and there may be more than one mechanism for the formation of bile pigments. Libowitzky & Fischer (61) have prepared an ester of the ferrichloride of α -hydroxyisocoproprophyrin I by the action of peroxide upon esterified pyridine coprohemochromogen. The former is a green pigment with absorption at 655 m μ (same as verdohemochromogen), is a precursor of bile pigments, and has an unruptured porphyrin ring. This work has led to the conclusion that Lemberg's verdohemochromogen is probably not an open-ring compound. Lemberg *et al.* (49) have taken issue with this deduction since biliverdin production from verdohemochromogen is readily demonstrated under much milder conditions than those found necessary for the transformation of the precursors synthesized in Fischer's laboratory. More recently Libowitzky (62) has carried out the full synthesis of coprobilirubin I from the tetramethyl ester of pyridine coprohemochromogen I. The intermediates isolated in the course of the synthesis included a green-pigmented derivative of the ferric chloride of α -oxy-coproprophyrin but no open-ring compounds.

Libowitzky (62) remarks upon the great difference in the effect of peroxide upon ferrous and ferric derivatives. Ferrous complexes of coproporphyrin esters yield green pigments. Ferric complexes, on the other hand, react only with very high concentrations of peroxide to form propentdyopents (oxydipyrrromethenes) (63). Pentdyopent is a naturally occurring substance (157). Treatment of iron porphyrin derivatives in alkaline solution with hydrogen peroxide rapidly results in the decolorization of the solution and quantitative liberation of the iron (64).

Holden & Lemberg (65) have discovered a striking difference in the spectra of iron porphyrin derivatives and bile pigment precursors. The so-called Soret or γ -band (maximum at 420 to 415 m μ) is absent in the open-ring derivatives. This is an important criterion and may be offered as proof of structure. Further evidence for the open ring in pyridine verdohemochromogen was obtained by condensing it with ammonia, thereby producing the corresponding derivative of an azaporphyrin (49).

Evidence of the natural occurrence of bile pigment precursors has been found. Barkan & Walker (66) have demonstrated that in human blood incubated for six hours at 37° C. a small simultaneous transfer of bilirubin and iron from cells to plasma occurs in 60 per cent of the samples examined. This is concluded from data upon the increase in bile pigment and iron above that found in nonincubated control samples. The observed rates of increase account for only a small fraction of the daily production of bile pigment. Lemberg, Lockwood & Legge (49) also have isolated bile pigments (biliverdin, 70 per cent, and biliviolin, 30 per cent) from erythrocytes. Their analyses indicate that less than 1 per cent of the red cell hemoglobin is of bile pigment nature (choleglobin). Bile pigment-hemoglobin can account therefore for only a fraction of the so-called labile or easily split iron of the blood.⁶ The presence of bile pigment-hemoglobin in the blood of rats dosed with sulfonamide drugs has been reported (67). A preliminary report by Lemberg *et al.* (68) indicates that one of the four prosthetic groups of catalase (from horse liver) is a chole-hemin, the others being protohemin. Also of pertinence is the finding of Yakushiji & Okunuki (69) that solutions of cytochrome-*a*, relatively free of other cytochrome components, become green upon reduction (strong maximum of absorption at 605 m μ). It is suggested by

⁶ Cf. p. 549.

these workers that cytochrome-*a* may be of verdohemochromogen nature. The purified preparation is said not to combine with carbon monoxide. This conclusion, both in the present work and in past reports upon cytochrome-*a*, appears to be based largely upon spectroscopic evidence. It may be important, therefore, to emphasize that the spectrum of cholehemochromogen does not reveal the combination with carbon monoxide (cf. 48), although a carbonyl derivative undoubtedly is produced.

Papers dealing with recent methods of preparation of bile pigments and analytical procedures of interest are cited in the bibliography (70 to 75). For the synthetic organic chemistry phase of the problem readers are referred to a review by Siedel (76).

Stercobilins and stercobilinogens.—The excreta (particularly feces) of subjects with hemolytic icterus have proved very useful as rich sources of material in the study of bile pigment metabolism. Fischer & Libowitzky (77) have isolated in large amount from this source bilirubin identical with that obtained from ox gallstones by extraction with boiling chlorobenzene (71). Besides bilirubin, stercobilinogen (identical with urobilinogen) and mesobilirubinogen IX_a were isolated by means of extraction with alcohol, followed by adsorption upon aluminum oxide and elution with ether. Freshly collected feces were found to be free of stercobilin (identical with urobilin) although this substance was produced rapidly as the feces stood exposed to air. It may be deduced that stercobilin originated from stercobilinogen by an oxidative reaction. However, all attempts to reduce stercobilin to stercobilinogen *in vitro* failed. Similarly, laboratory methods of oxidation fail to accomplish the reversal of stercobilinogen to products resembling bilirubin.

METALLOPORPHYRINS

Nomenclature.—Clark (1, 78) and the writer (79) independently have introduced for derivatives of metalloporphyrins a similar new nomenclature, which is receiving adoption (158d). In this terminology, which shall be employed here, the various compounds are described as derivatives of ferri- or ferroporphyrin, using a suitable prefix to indicate the type of porphyrin, for example, ferri- or ferroporphyrin. The compound variously designated as cyanhematin, hematin dicyanide, cyanide parahematin, and oxidized cyanide hemochromogen is now described by the term dicyanide ferriprotopor-

phyrin. This nomenclature appears to have the merits of simplicity and general applicability. It avoids the ambiguities which have arisen in the use of the term hemochromogen by various workers.

Hemins.—The term hemin, as usually employed, represents the chloride of ferriprotoporphyrin IX. Fischer's method for the preparation of this hemin from defibrinated beef blood has been published only recently in full detail (80). Purification of the isolated product is accomplished by solution of the dried crystal crop in pyridine-chloroform and recrystallization from hot glacial acetic acid-sodium chloride solution.

Due to its solubility characteristics, restrictions have been imposed upon the study of hemin in solution. Such studies are usually carried out in alkaline solutions (pH 11 or greater). Under these conditions the equilibrium operating in the formation of metalloporphyrins [porphyrin + reduced metal ion \rightleftharpoons reduced metalloporphyrin + $2H^+$ (4, 28)] is shifted far to the right and may be neglected (1) as a complicating factor in the study of other equilibria such as those involved in the coordination of metalloporphyrins with nitrogenous bases or with hydroxyl ion. As a result of the latter equilibrium, ferriprotoporphyrin hydroxide is formed from hemin in alkaline solution (3). The data of Clark & Perkins (5) obtained by photoelectric spectrophotometry fit closely the assumed equation: ferricoproporphyrin hydroxide + H^+ \rightleftharpoons ferricoproporphyrin + water. This reaction is therefore one of first order with hydrogen ion, with best fit of data found at $pK = 7.44$. In glacial acetic acid, the splitting of mesoporphyrin from ferromesoporphyrin has been demonstrated by Vestling (4). Hence, under these conditions the equilibrium functioning in metalloporphyrin formation (see above) may be shifted to the left. Knowledge of this fact has been utilized in the synthesis of iron porphyrins (4) by employing a not-too-acid menstruum, namely a glacial acetic-acetate mixture. Clark and associates [see Davies (3)] have found it convenient to postulate for monomeric hemins in alkaline solution a structure in which one hydroxyl and one water group, besides the four pyrrol nitrogens, coordinate with the iron. This satisfies the usual rule of hexa-coordination in iron complexes.⁷ Morrison & Williams (81) have concluded from titration data, obtained under conditions unfortunately complicated by the presence of a solid phase, that three equivalents of alkali are required in the formation of disodium ferri-

⁷ Cf. p. 546 and footnote to Table I, p. 561.

hemate (the disodium salt of ferriprotoporphyrin hydroxide). This is in harmony with Davies' postulated structure of hemin in alkaline solution (3). According to Morrison & Williams, the three equivalents of alkali simultaneously neutralize the carboxyls of the two propionic groups and hydrolyze the chloride.

The original determination of the molecular weight of hemin in boiling pyridine led to the conclusion (82) that it was monomolecular in this solvent. A suggestion by Anson & Mirsky (83) that hemin may be aggregated in solution has been supported recently by evidence from three independent sources: sedimentation equilibrium data upon alkaline and acid solutions in centrifugal fields (84); spectra of hemin in such solvents as hydrochloric acid-alcohol (85); and rate of diffusion of proto- and mesohemin in alkaline solutions through sintered glass membranes (86). Gralén's sedimentation measurements (84) indicate that hemin in alkaline solution is very polydisperse, with the molecular weight magnitudes of the aggregates varying from 30,000 to 60,000. The diffusion measurements yield a value of 50,000. In acid even larger aggregates are present (84). A satisfactory explanation of the phenomenon of aggregation as applied to hemin and its hydroxide has not been advanced. The development of a unified theory should perhaps also explain the apparently paradoxical behavior of certain nitrogenous derivatives of hemin. From careful electrometric titration measurements Davies (3) has concluded that nicotine ferri- and ferriprotoporphyrin behave as dimers in aqueous solution but as monomers in 47 per cent aqueous alcohol. Hemin, on the other hand, may be aggregated in acid alcoholic solution (85). Davies' data suggest that the two coordination centers in the dimerized systems are intimately linked energetically, and he therefore has considered the possibility that a hydroxyl bridge may link the two iron atoms (3). It may be pointed out that fundamental structural differences exist between hemin, its hydroxide, and their nitrogenous derivatives. The latter are essentially covalent in character; the former, essentially electrovalent.⁸

The catalytic destruction of hemin and hemoglobin through a coupled oxidation of the pigments with either linoleic or linolenic acids in the presence of air at 38° C. has been described by Haurowitz *et al.* (87). In this reaction iron is liberated and the prosthetic group is broken down to colorless units, without measurable production of

⁸ Cf. Table I, p. 561.

porphyrins or bile pigments. This process is proposed (87) as another possible catabolic pathway for hemoglobin (Figure 1c) in which the unsaturated essential fatty acids play a functional role.

Coordination complexes of metalloporphyrins and nitrogenous bases.—The knowledge of naturally occurring substances such as cytochrome-*c*, cytochrome oxidase, the Pasteur enzyme, catalase, myoglobin, and hemoglobin, which belong to this group, has been advanced by a study of simpler nitrogenous derivatives of hemin created in the laboratory. The contributions of Clark and his students, Taylor, Davies, and Vestling (1 to 5), who have undertaken a systematic electrometric and spectrophotometric exploration of systems involved in the coordination of metalloporphyrins and nitrogenous bases, are of outstanding importance and serve as a model for future effort in this field. Reference has already been made to certain phases of the work, and only general considerations and conclusions will be discussed in the following paragraphs.

At the outset, orientation may be afforded by once again referring to the postulated structure (3) of monomeric ferriporphyrins in alkaline solution as a complex containing one molecule of water and one hydroxyl ion in the coordination shell. In ferroporphyrins a structure with two molecules of water in the coordination shell best accounts for the observations (3). These structures are consonant with the following deductions: Neutral bases (pyridine, nicotine) compete easily with the two water molecules in the shell of a ferroporphyrin, but less easily with the one molecule of water in ferriporphyrin hydroxide. This accounts for the recognized greater affinity of nitrogenous bases for ferrohematin. Neutral bases, under usual conditions, do not compete with the hydroxyl ion in the shell of ferriporphyrins, but cyanide ions do so readily. This follows from the fact that the potentials and spectra of the cyanide complexes are invariant with pH in contrast to the pH-dependent potentials and spectra of neutral base derivatives.

The complexity of a system containing metalloporphyrin and a coordinating nitrogenous base has been revealed to be exceedingly great. As Clark has pointed out (78, 88), such a system embraces a set of integrated processes: the reversible oxidation and reduction of the metal ion component; the independent reversible associations of the oxidized and reduced metalloporphyrin with the nitrogenous substance; the reversible association of one or more components with hydrogen or hydroxyl ions (see above); and, in some cases, as has already been discussed, the aggregation of metalloporphyrins or their

derivatives. The independent association of oxidized and reduced metalloporphyrins with nitrogenous substances accounts for the observed change in potential (increase in the cases studied) when a nitrogenous base is added to a fixed mixture of the oxidized and reduced species of the metalloporphyrin. This change in potential indicates a difference in the free energies of association of oxidant and reductant, a factor which may prove of interest in the ultimate analysis of the roles of natural metalloporphyrin complexes in catalysis (1). From the practical standpoint the change in potential, which is a function of K_O/K_R , the ratio of the respective dissociation constants of the oxidized and reduced nitrogenous metalloporphyrins, permits electro-metric titration to be used as a criterion of the association equilibrium. The association may also be followed spectrophotometrically. The two methods supplement each other (2, 3, 5). Experimental difficulties often prevent the securing of accurate data at all points of the typical symmetrical association curves. Rectification and testing of such curves is accomplished by the application of the impersonal graphic approximation method of Reed & Berkson (89).

The following, in brief résumé, are some of the conclusions of Clark *et al.* as to the coordination of metalloporphyrins with nitrogenous substances: (i) two molecules of pyridine (or nicotine, or cyanide) associate with manganomesoporphyrin, ferroprotoporphyrin, or ferrocoproporphyrin. (ii) Ferroporphyrins coordinate better than ferriporphyrins with nitrogenous bases. (iii) The evidence is inconclusive as to whether one or two molecules of neutral bases coordinate with ferrimeso- and ferriprotoporphyrin. However, two molecules of pyridine probably associate with manganimesoporphyrin, and spectrophotometric evidence suggests that ferricoproporphyrin "violates the rule" of hexa-coordination and associates with two pyridines and one hydroxyl ion.⁹ (iv) Ferricoproporphyrin (and presumably other ferriporphyrins) coordinates with two molecules of cyanide.

The writer (79) has found a new, general reaction of cyanide with nitrogenous ferriprotoporphyrins, which must be distinguished from the reaction of cyanide with ferriporphyrins themselves, mentioned above. The cyanide derivatives of the nitrogenous ferriprotoporphyrins behave spectroscopically like analogues of cyanmethemoglobin. The derivatives are formed in unusually low concentrations of cyanide, only one mole of the latter per iron atom present being required for

⁹ Cf. footnotes to Table I, p. 561.

complete reaction. Biological applicability may be suggested for this reaction from the standpoint of the mechanism of cyanide poisoning.

Zeile & Gnant (86) have deduced from spectrophotometric measurements that four molecules of ferroproto- or ferromesoporphyrin coordinate easily per equivalent (16,400 mol. wt. unit) of denatured globin, complete reaction presumably being attained at a concentration ratio of four moles of the ferrohemin to one equivalent of the protein. If each ferrohemin molecule coordinates with two nitrogen atoms, this would account for a total of thirty-two nitrogens with which sixteen hemin molecules could coordinate in the denatured protein derived from one mole of hemoglobin. While Zeile & Gnant do not make the suggestion, it is of some interest to point out that globin contains thirty-two to thirty-three histidine residues (90) which may appropriately be considered as possibly involved in this coordination.

Methemalbumin is presumably an association product of ferrihemin and plasma albumin (91). This pigment forms in plasma incubated with solutions of oxyhemoglobin and is thought by Fairley (91) to be similar to a pigment found in the plasma of patients with black-water fever. The spectra of cobaltiprotoporphylin, cobaltoprotoporphylin, their globin, cyanide, and imidazole derivatives, and the absorption constants upon an iron basis (64) of pyridine ferroproto-, ferromeso-, and ferrocoproporphyrin have been reported respectively by Holden (92) and Drabkin (93).

Hemoglobin and myoglobin.—From the analysis of globin prepared from hemoglobin Birkofer & Taurinš (94) conclude that characteristic species differences exist in the molecular ratios of methionine to cystine. A comparison (95) of the content of certain amino acids in horse hemoglobin and myoglobin has not revealed striking differences between the two proteins. The similarity of the two globins (exclusive of molecular weight) is also suggested by the identical spreading properties of their monolayer films (96).

Conclusions of considerable significance have come from the data of Wyman *et al.*, secured by an application of physical techniques, which included differential acid-base titration of ferroheminoglobin and oxyhemoglobin (97) and of oxyhemoglobin against ferrihemoglobin (98), titration of oxyhemoglobin over the range pH 4 to 10 by a thermal method (99), and a study of the relationship of the heat of oxygenation and base-binding groups (100). As a result of this work the imidazole group of histidine may be considered to be involved both in the oxygenation equilibrium and in the union of globin and

the prosthetic group. The identification of this group follows from its activity in the range pH 5.5 to 8.5 (99), the similarity of the heat of oxygenation (related to the heat of dissociation of base-binding groups which interact with oxygen) and the heat of dissociation of the imidazole group (100), and the constancy of potential obtained by Taylor & Hastings (101) for ferrohemoglobin-ferrihemoglobin at pH 5.0 to 6.0. In ferro- and ferrihemoglobin three "heme-linked" or heme-interacting acid groups have been revealed. Applying Coryell & Pauling's concepts (102), the groups are respectively the imidazolium ion, pK_1 , displaceable by oxygen or carbon monoxide and restrained from strong electrostatic coordination with the iron by the presence of the second imidazole group whose imino nitrogen is responsible for pK_2 , and the iron atom itself, which may add an hydroxyl ion (as in ferrihemoglobin hydroxide), responsible for pK_3 . That ferrihemoglobin participates in the equilibrium, $\text{ferrihemoglobin} + \text{OH}^- \rightleftharpoons \text{ferrihemoglobin hydroxide}$, was established spectrophotometrically by Austin & Drabkin (103), with $pK_3 = 8.12$ at 0.1 ionic strength. This value has been confirmed both by magnetometric (104) and differential acid-base titration (98). Wyman & Ingall's values (98) for pK_1 and pK_2 at 25° C. and 0.16 ionic strength are $pK_1 = 5.25, 5.75, 5.75$, and $pK_2 = 7.93, 6.68, 6.68$ for ferro-, oxy-, and ferrihemoglobin, respectively. The apparent dissociation ($pK = 6.65$) in ferrihemoglobin disclosed in the variation of the midpoint potential in the data of Taylor & Hastings (101) upon the ferrohemoglobin-ferrihemoglobin system may be reconciled with the data by the other methods without postulating the formation upon oxidation of a new ionizable group in addition to that responsible for pK_3 (98, 102).

The application of spectrophotometry to turbid biological media has been initiated by Drabkin & Singer (105) in a quantitative study of whole blood, utilizing a cuvette of 0.007 cm. depth (106) and suspensions of erythrocytes. The extinction due to pigment, ϵ_p , was found to be derived accurately from the empirical equation developed from the data, $\epsilon_p = \epsilon_t - \epsilon_s/f_1 (Nd)$, where ϵ_t is total extinction, ϵ_s , scattering extinction (also a function, f_2 of Nd), N , the number particles per c.mm., and d , the depth in centimeters. In the wave length range 630 to 500 m μ the spectra of intracellular oxyhemoglobin and intracellularly produced ferrihemoglobin and ferrihemoglobin cyanide were deduced to be identical with the spectra of solutions of these pigments. The pH dependency of the spectrum of ferrihemoglobin (103) permitted the deduction of the intracellular pH of the erythrocytes.

Adams (107) has made the interesting observation that the γ -band (420 to 415 m μ) is absent in the spectra of red cell suspensions and has ascribed the phenomenon to a combination of hemoglobin and "stromatin." Since the absence of the near ultraviolet absorption band is characteristic of open-ring compounds (65), Adams's observation could imply the existence of profound differences in the structure of intracellular hemoglobin. This is improbable. Keilin & Hartree (108) have confirmed the absence of the γ -band in the spectra of erythrocyte suspensions but find that "stromatin" definitely is not responsible. This curious optical phenomenon can be duplicated by the dispersion of oxyhemoglobin in suitable preparations of paraffin oil (108). Matthes & Gross (109) have described continuous recording techniques for the study of blood *in vivo* which utilize the differences in light absorption of ferro-, oxy-, and carbonmonoxyhemoglobin in the red and infrared spectral regions.

The finding of so-called "inactive hemoglobin" in blood and its relationship to "labile iron" and bile pigment precursors (50) presents a perplexing problem. It is well established that identical values of oxygen and carbon monoxide capacity by the usual techniques (110) are obtained from blood samples. However, it has been found that the carbon monoxide capacity by the Van Slyke & Hiller procedure (110) may be greater by approximately 5 per cent in both human (111) and bovine (112) blood samples after the samples are treated with hydrosulfite than before such treatment. This finding appears to suggest that the average blood sample may contain approximately 5 per cent of a ferriporphyrin complex capable of combining with carbon monoxide after reduction. The "inactive hemoglobin" does not appear to be solely ferrihemoglobin (111). From the standpoint of satisfactory interpretation, it is unfortunate that it is not possible (due to formation of strong oxidants) to carry out comparable oxygen capacity determinations after treatment of blood with hydrosulfite. In the course of his studies the writer has not found significant discrepancies in the routine spectrophotometric determination of "total pigment" as ferrihemoglobin cyanide and oxyhemoglobin in several hundred samples of dog blood. The question has been raised as to the identity of "labile iron" or easily split iron (ESI) of blood and "inactive hemoglobin" (50). Legge & Lemberg (51) confirm Barkan & Schales's finding (50) that ESI is of the order of 5 per cent of the total iron of blood, and that only one third, the ESI_{∞} fraction, is easily split by acid in the presence of carbon monoxide, whereas two thirds

($ESI_0 - ESI_{\infty}$) are split only in the presence of oxygen. However, Legge & Lemberg offer evidence that the larger ($ESI_0 - ESI_{\infty}$) fraction of the "labile iron" is an artifact arising from the oxidation of the prosthetic group by the oxygen evolved from hemoglobin in the presence of acids. Hence no more than approximately 2 per cent of the total iron of blood may be considered to represent preformed ESI.

Interest has continued in the study of the denaturation of hemoglobin by various reagents such as urea (113, 114), acetamide (113, 114), urea and alkali (115), and the detergent Duponal P. C. (116). Hemoglobin reconstituted from hemin and globin, while homogeneous and very similar in most physical properties to native hemoglobin, shows slight evidences of difference. The synthetic product, in contrast to the natural, precipitates upon electrodialysis, has a displaced electrophoretic mobility curve (84), and is less resistant to alkali (117). Studies of the metabolism of hemoglobin, using radioactive iron as a tracer element, have been initiated in Whipple's laboratory (118, 119). Radioactive iron in the form of ferrous gluconate, fed to anemic dogs, is demonstrated to be present in the circulating red cells within four hours after administration. The exchange of radioactive iron between cells and plasma cannot be demonstrated *in vitro*, nor can radioactive iron be transferred to hemoglobin in solution (119). Readers are referred to reviews of carbon monoxide poisoning (120) and myoglobin (121).

Cytochromes.—The contributions of Theorell (122) and Theorell & Åkesson (123, 124, 125, 126) have greatly extended the knowledge of the chemistry and constitution of pure cytochrome-*c*. The pigment products obtained by the Keilin & Hartree procedure (127) vary in iron content from 0.22 to 0.34 per cent (93, 123). Such preparations were subjected to prolonged electrophoresis at suitable pH (123), thereby yielding pure, homogeneous cytochrome-*c* with an iron content of 0.43 per cent (mol. wt. = 13,000). Of more pertinent present interest are the six atoms of sulfur and three molecules of histidine per atom of iron in the chromoprotein. Two of the sulfur atoms belong to two cysteine molecules probably joined by thio-ether linkages to the two vinyl rests of the cytochrome-*c* prosthetic group. This deduction is based upon Theorell's original formulation of the constitution of "porphyrin *c*" (122), which has now been verified (128) upon the porphyrin prepared from cytochrome-*c* under conditions which apparently preclude its being an artifact.

The remarkable pH stability range (pH 1 to 13) of cytochrome-*c*

has permitted a more adequate study to be made of the pH dependence of spectra (124) and magnetic susceptibility (126) than is possible with hemoglobin. In ferricytochrome-*c* by these means five heme-linked acid groups have been uncovered (124, 125, 126), $pK_1 = 0.42$, $pK_2 = 2.50$, $pK_3 = 9.35$, $pK_4 = 9.85$ (spectroscopically "inoperative"), and $pK_5 = 12.76$. Spectroscopic analogy permits the deductions that the species in the region pK_1 are hemin-like and that those in the region pK_2 correspond to acid ferrihemoglobin (124). The constants pK_3 and pK_4 are interpreted from the differential acid-base titration of ferro- and ferricytochrome to represent imino groups of two abnormally placed histidine residues (125), which are deduced to function in the coordination of the protein and the prosthetic group. In contrast to hemoglobin, both imidazole groups coordinate strongly in cytochrome-*c*, and besides the protein is bound to the prosthetic group also by thio-ether linkages mentioned above. Thus, Theorell pictures hemin-*c* as "built into a crevice of the protein molecule" (126). It is thereby protected from autoxidation and from coordination with carbon monoxide and cyanide in the physiological range of pH.

The absorption constants of ferrocytochrome-*c* upon an iron basis have been established (93), and the analysis of the spectrum has been reported (129). Fujita *et al.* (130) have published the results of extensive analyses of the cytochrome-*c* content of various tissues. Ball & Meyerhof (131) have demonstrated the presence of cytochrome-*c* and succinic acid dehydrogenase in the muscles of several species (horse-shoe crab, lobster) whose circulating blood pigment is hemocyanin, and they have drawn the interesting inference that tissue respiration in these forms is probably similar to that in mammals. The evidence for the existence of a cytochrome-*c*₁ component (132) does not appear impressive. Reference already has been made to work (69) on the isolation of cytochrome-*a*. A full account of the criteria for the differentiation of recognized cytochrome components, *c*, *b*, *a*, *a*₁, *a*₂, and *a*₃, has been furnished by the researches of Keilin & Hartree (133). Cytochrome-*a*₃, discovered by these investigators, possesses many properties usually identified with cytochrome oxidase.

Pasteur enzyme.—The essential independence of respiration and glycolysis in mammalian tissue was suggested strongly by the experiments of Laser (134) upon surviving retina. Such an independence implies the probable existence of a catalyst distinct from cytochrome oxidase and concerned in the catalysis of the Pasteur reaction (re-

straint of glycolysis by oxygen). Stern & Melnick have named this postulated catalyst "Pasteur enzyme," and have measured its relative photochemical spectrum (135, 158b). The spectrum of the Pasteur enzyme does not differ strikingly from that of cytochrome oxidase obtained by similar means, but statistical testing suggests that the difference may be real (136).

Catalase.—The report by Lemberg and colleagues (68) that catalase contains a changed hemin group has been verified by Sumner *et al.* (137). In beef and horse liver preparations by a new method these investigators find that the activity is inversely proportional to the ratio of so-called blue iron (iron from changed prosthetic groups) to hemin iron. In a study of the significance of rate of diffusion in manometric measurements, Roughton (138) has concluded that the factor of diffusion must be considered in rapid reactions such as the liberation of oxygen from peroxide by catalase preparations. Greenstein *et al.* (139) have found that rats bearing induced hepatic tumors show a large drop in liver catalase activity, which is promptly restored to normal levels following excision of the tumor.

THE MAGNETIC CRITERION FOR BOND TYPE IN THE STUDY OF THE MOLECULAR STRUCTURE OF DERIVATIVES OF FERRI- AND FERROPORPHYRINS

The determination of the molal paramagnetic susceptibility, χ_m , has been applied with notable success in Pauling's laboratory to the study of the molecular structures of hemoglobin, hemin, cytochrome-c, and many of their derivatives. The measurements have permitted the calculation of the number of unbalanced or unpaired electrons in the various molecules studied, and from this nonrigorous quantum-mechanical deductions as to the bond type present have been drawn (140).

Theory.—With reference to their susceptibility to an applied magnetic field, substances may be divided into two classes: (i) substances which are rendered diamagnetic (i.e., repelled by a magnet), and (ii) substances which are rendered diamagnetic, but are also inherently paramagnetic (i.e., attracted by a magnet).

The diamagnetic polarization results from an accelerating influence of the magnetic field upon the electrons of all substances and has not been of value in the investigation of the nature of the chemical bond. Paramagnetic polarization, an inherent characteristic of certain substances, is associated with the presence in these substances of

atoms, ions, or molecules containing unpaired electrons. The spin magnetic moments, μ_s , of the unpaired electrons (the dominant factor in the case of compounds of iron) and the moments due to their orbital motion contribute to a resultant unbalanced permanent magnetic dipole moment, μ , responsible for paramagnetic polarization. These permanent magnetic dipoles orient themselves with the magnetic field, the degree of stable orientation with the field being a function of the temperature. In ideal systems where the interaction between the dipoles is small, paramagnetic polarization is inversely proportional to the absolute temperature. Deductions as to molecular structure are based upon the measurement of the paramagnetic contribution to the magnetic susceptibility. This is accomplished, as described below, by the fact that the force (of attraction) of the oriented permanent magnetic dipoles towards the magnet is directly proportional to the value of the molal paramagnetic susceptibility, χ_m , and may be measured gravimetrically.

When measured in units of Bohr magnetons the permanent magnetic dipole moment, μ , is obtained from the equation

$$\mu = 2.84\sqrt{\chi_m T}, \quad (\text{I})$$

where 2.84 represents $\sqrt{3k/N}$, k being Boltzmann's constant and N Avogadro's number, and T is the absolute temperature.

In the cases of inorganic iron ions and organic complexes of iron, which are of main present interest, the orbital motion (concerned with the moment μ_o) of the unpaired electrons is conveniently nearly quenched, and the spin moment, μ_s , approximates in magnitude the total permanent magnetic dipole moment, μ . μ_s is a function of the number, n , of unpaired electrons and theoretically may be calculated from the equation

$$\mu_s = \sqrt{n(n+2)} \text{ Bohr magnetons.} \quad (\text{II})$$

For one to five unpaired electrons the above equation yields the calculated values 1.73, 2.83, 3.88, 4.90, and 5.92. From determined values of χ_m the values of μ are derived from equation I. By substitution of these values (unchanged when $\mu_o = 0$ or appropriately corrected) for μ_s in equation II the number, n , of unpaired electrons in the compound under investigation is obtained.

Notation and definitions.—The electronic configurations of the atoms hydrogen, helium, neon, and iron are respectively $1s$, $1s^2$, $1s^2 2s^2 2p^6$, and $1s^2 2s^2 2p^6 3s^2 3p^6 3d^6 4s^2$. In this system of notation the

numerals (1, 2, 3, 4) before the letters (*s*, *p*, *d*) represent respectively the electron shells (*K*, *L*, *M*, *N*) surrounding the nucleus of the atom and give the values of the total quantum number *n*. The letters *s*, *p*, *d*, with respective values of 0, 1, 2, represent the orbitals in the particular shell and the azimuthal quantum number *l*, with a maximum value of $l = n - 1$. The number of possible orbitals in a shell is given by n^2 . Thus there is one in the *K* shell, four in the *L* shell (one *s* and three *p* orbitals), nine in the *M* shell (one *s*, three *p*, and five *d* orbitals), and so on. The *s* orbital has the lowest energy value, is most eccentric, penetrates most deeply into the core (nuclear region), and is the most stable. The stability sequence of the orbitals is given by $ns > np > nd$, etc. The superscripts to *s*, *p*, *d* give the number of electrons in the particular orbit. The summation of these numerals, one for hydrogen, two for helium, ten for neon, and twenty-six for iron, gives the total number of electrons.

With reference to the number of electrons associated with a particular orbital, the following rules may be given: (i) All the shells need not be associated with their full theoretical complement of electrons; some orbitals may be unused in a particular atom or molecule. (ii) A single orbit may be occupied by a single, unpaired electron, or by no more than two electrons, with the additional requirement in this case that the two electrons must be paired, i.e., they must have opposed spins which mutually neutralize their magnetic moments (Pauli's exclusion principle). (iii) An electron may be shared by two atoms, or more commonly a pair of electrons may be shared. Such shared electron pairs are at the basis of formation of covalent or electron-pair bonds (see p. 556). (iv) The electrons tend first to occupy the more stable orbitals, as 1*s*, 2*s*, 3*s*, doubly as paired electrons. When several orbitals of approximately the same energy are available, as in the case of the three 2*p* orbitals, the tendency is to occupy these orbitals singly, as unpaired electrons.

In a magnetic field the unpaired electrons orient themselves with the field and are responsible for the paramagnetic contribution. In consequence of the single unpaired electron in the 1*s* orbit of the hydrogen atom and the two paired electrons (as required by the exclusion principle) in the 1*s* orbit of the helium atom, monatomic hydrogen gas is paramagnetic while helium is diamagnetic. Referring to the respective electronic configurations, it is evident that the neon atom has its full complement of ten paired electrons in the five orbitals

of the *K* and *L* shells, while the iron atom has a full complement of paired electrons in all its orbitals except the *3d* (of the *M* shell). There are five *3d* orbitals. These may be occupied by five single unpaired electrons or by a full complement of ten paired electrons. The configuration of the iron atom indicates the presence of six electrons in the *3d* orbitals. On the basis of the principles of electron distribution which have been mentioned, it follows that four of these electrons are unpaired and occupy singly four different *3d* orbitals, while two of the electrons are paired and together occupy a fifth *3d* orbital.

The electronic configuration of the ferric ion (Structure A), with a total of twenty-three electrons about the iron nucleus, is $1s^2 2s^2 2p^6 3s^2 3p^6 3d^5$. There are five unpaired electrons occupying the *3d* orbitals. The presence of five unpaired electrons gives the ferric ion the large magnetic moment of 5.92 Bohr magnetons (see equation II). Indeed this is a maximum value, for no more than five unpaired electrons can be present in the *3d* orbitals. When additional electrons are crowded into these orbitals, pairing is required. Thus, complexes of iron which have high magnetic moments of the order of magnitude of that for the ferric ion are deduced to be essentially ionic in character. Ionic bonds between atoms or groups are electrostatic bonds and result from Coulomb attraction of the excess electric charges of oppositely charged ions. For complexes of iron the bonds are described as essentially ionic to indicate that extreme ionic character is not assumed. Weak unstable covalent bonds could be present in the *4s*, *4p*, or higher orbitals without disturbing the electron configuration in the *3d* orbitals.

Upon the addition of cyanide ion (CN^-) to the strongly paramagnetic ferric ion in solution the ferricyanide ion, $[\text{Fe}(\text{CN})_6]^\equiv$ (Structure B), is formed with an accompanying immediate drop in the paramagnetic susceptibility, χ_m . When the reaction is complete, χ_m corresponds to a magnetic moment, μ , of 1.73 Bohr magnetons (equation I). This indicates the presence of one unpaired electron in the complex ferricyanide ion (equation II). It is deduced that union with six cyanide groups has had the effect of crowding the five unpaired electrons in the five *3d* orbitals of the ferric ion into three of these orbitals, thereby permitting the presence of but one unpaired electron in the ferricyanide ion. In the present discussion of the electronic configuration of this complex ion we need only consider, besides the twenty-three electrons of the ferric ion, six additional electrons concerned in the bonds of the six cyanide groups with the iron atom.

Since only one unpaired electron is present in the structure (and since this electron has already been accounted for), all other available orbitals (i.e., orbitals which may be used for bond formation) must contain paired electrons. The six most available orbitals in this case prove to be the remaining two $3d$, one $4s$, and three $4p$. Each of these orbitals accommodates a pair of shared electrons, one contributed by the iron atom, the other by a cyanide group. Of the ferric complexes $[\text{Fe}(\text{CN})_6]^\equiv$ represents structurally the opposite extreme from Fe^{+++} and is described as essentially covalent in character.

From the chemical standpoint this is important: In changing from ionic Fe^{+++} to octahedral covalent $[\text{Fe}(\text{CN})_6]^\equiv$ six new chemical bonds were produced. The bonds correspond to the classical valence bonds and are now designated "covalent" or "electron-pair" bonds. Such bonds are formed by the sharing of paired electrons by two atoms or groups. The neutralization of the spin magnetic moments of the electrons as they are paired accounts for the decrease in the magnetic moment and renders the determination of paramagnetic susceptibility a valuable tool in the study of structures such as the above.

Three pertinent points may be added. (i) The valence shell is the outermost shell. However, in the transition group atoms (iron, palladium, and platinum groups) the d orbitals of the next inner shell have approximately the same energy values as the s and p orbitals of the valence shell, and for these atoms and complexes derived from them the d orbitals (if unoccupied by unshared electrons) are very important in covalent bond formation. The covalent bonds of octahedral iron complexes appear, as exemplified by the case of the ferricyanide ion, in the $3d$, $4s$, and $4p$ orbitals. In the notation of the bond it is customary to omit the number referring to the shell, and to give superscripts denoting the number of pairs of shared electrons present (equivalent to the number of electron-pair bonds, or one half the number of electrons in the particular orbitals). Thus the covalent bonds of the ferricyanide ion are described as d^2sp^8 hybrid bonds (i.e., orbitals of two shells are utilized in bond formation). (ii) The strength of covalent bonds is a function of the resonance energy of the two shared electrons concerned in the bond, and their direction is determined by preference for the position in which the "overlapping" atomic orbitals are concentrated. Mathematical analysis leads to the conclusion that the most stable configuration for iron complexes with six equivalent bond orbitals of nearly maximum possible strength, as in the ferricyanide ion, is one in which the covalent bonds are directed

towards the corners of a regular octahedron. Accordingly, the covalent bonds of greatest present interest may be described fully by the designation "octahedral covalent d^2sp^8 bonds." (iii) The primary rule of resonance requires that resonance can occur only between structures which have the same number of unpaired electrons. Thus, resonance is impossible, and continuous transition between essentially ionic structures, such as Fe^{+++} , and essentially covalent structures, such as $[\text{Fe}(\text{CN})_6]^\equiv$, cannot occur. Discontinuity must be present in the transition from one type to the other.

The ferrous ion, Fe^{++} (Structure C), has a total of twenty-four electrons (one more than the ferric ion). The additional electron is paired in one of the $3d$ orbitals, leaving four unpaired electrons, in contrast to five for the ferric ion. As might be expected, in the ferrocyanide ion, $[\text{Fe}(\text{CN})_6]^\equiv$ (Structure D), the phenomenon of crowding the unpaired electrons into the available $3d$ orbitals (already discussed for the ferricyanide ion) results in a configuration in which all the orbitals have their full complements of paired electrons. Accordingly, the magnetic moment is zero, and $[\text{Fe}(\text{CN})_6]^\equiv$ is diamagnetic. [For a further exposition of theory readers are referred to Pauling's excellent text (140)].

The four structures, Fe^{+++} (A, with five unpaired electrons), $[\text{Fe}(\text{CN})_6]^\equiv$ (B, with one unpaired electron), Fe^{++} (C, with four unpaired electrons), and $[\text{Fe}(\text{CN})_6]^\equiv$ (D, with no unpaired electrons), may be considered as structural prototypes of most of the various hemin and hemoglobin derivatives studied by Pauling, Coryell, and colleagues, and of cytochrome-*c* and its derivatives studied by Theorell. For example, the magnetic moments (calculated from the determined values of χ_m) correspond to five unpaired electrons per iron atom in ferriprotoporphyrin (oxidized hemin), ferrihemoglobin (acid methemoglobin, pH 5.9), and ferricytochrome-*c* II, and to four unpaired electrons per iron atom in ferroprotoporphyrin (reduced hemin) and ferrohemoglobin (reduced hemoglobin). The iron atoms of these complex structures are deduced, on the basis of their magnetic behavior, to retain the essentially ionic configurations of either the ferric (A) or the ferrous (C) ions. Ionic bonds accordingly are assumed to exist between the iron atom and the four adjacent nitrogen atoms of the porphyrin in the above complexes (which may be designated Class I). On the other hand, the determinations of χ_m lead to a calculation of one unpaired electron per iron atom in ferrihemoglobin cyanide (cyanmethemoglobin) and ferricytochrome-*c* cyanide, and to

zero unpaired electrons (diamagnetic) in oxyhemoglobin, carbon-monoxymoglobin, and several nitrogenous derivatives of ferroprotoporphyrin (reduced hemochromogens). With respect to the electronic configurations of their central iron atoms these complexes may be regarded as structural analogues of the simpler inorganic complexes of iron, either of the ferricyanide (B) or of the ferrocyanide (D) ions. Hence each iron atom of complexes of this type (which may be designated Class II) is deduced to be bound not only to the four adjacent pyrrolic nitrogen atoms but also to two other atoms by essentially covalent bonds. As in the simpler complexes the six bonds are of the octahedral (d^2sp^3) type. It should be pointed out that in such structures the iron atom has been considered to be effectively space-saturated with reference to bonding, the structures being hexacoordination complexes. [However, it has been mentioned already that exceptions to this rule may exist among the hemin complexes, as originally postulated by Clark *et al.* (3, 5).]

The change from the structure of ferrihemoglobin to that of ferrihemoglobin cyanide has an apparent close counterpart in the change from ferric ion to ferricyanide ion. On the other hand, the profound structural change which reduced hemoglobin undergoes upon oxygenation and the structural difference between ferroprotoporphyrin (ferrohememin) and its nitrogenous derivatives were unexpected and had no counterparts in the simpler complexes of iron.

Method.—The technique of measuring paramagnetic susceptibility used in Pauling's laboratory (104, 112, 141) was devised in 1889 by Gouy (142) and has been described more fully by Shaffer & Taylor (143). The principle is that of "magnetic pull balanced by gravitational pull." The weight of a vertical glass cylinder containing the substance, suspended from one arm of a balance, is measured before and after the application of a strong magnetic field to one end of the cylinder, which is sufficiently long so that its other end may be considered to be in zero magnetic field. The difference in weight, Δw (in milligrams), when the exciting current of the electromagnet is on and off, is the force due to the magnetic field acting on the cylinder. As has been said already, this force is directly proportional to the susceptibility.

The following details may permit a better visualization of the technique: (i) The cylindrical tubes employed are 30 cm. long, 1.8 cm. in internal diameter, and are divided into an upper and lower chamber by a glass partition. The magnetic field is applied in the region of the

partition so that the force acts relatively symmetrically upon both chambers. The solution to be measured is placed in the upper chamber, the volume being of the order of 25 to 30 ml. In the lower chamber is placed the solvent (usually water). Assuming that both halves of the cylinder have identical magnetic properties (which is not necessarily the case), the arrangement is such that if both chambers are filled with the same paramagnetic or diamagnetic solution, the Δw should approximate zero. This follows from the fact that orientation with or against the magnetic field will produce, under these conditions, equal and opposite effects. If, however, a solution with measurable paramagnetic contribution is placed in the upper chamber while the lower chamber accommodates the relatively diamagnetic solvent, an appreciable positive Δw may be measured when the applied force is sufficiently strong. The arrangement is such that in this case the diamagnetic influence of solvent is balanced out (by opposite effects in the two chambers), and the paramagnetic orientation in the upper chamber results in the downward pull on the cylinder. The cylinders are calibrated by the determination of the susceptibility of water against air or of air against water, and they are corrected for relative magnetic asymmetry by determination of water against water. In determinations of absolute susceptibility experimentally obtained corrections are applied for the diamagnetism of added reagents and for dilution. Once the essential diamagnetism ($\mu = 0$) of carbonmonoxyhemoglobin (144) and the absolute paramagnetic susceptibility of ferrohemoglobin (per hemin unit) [$\chi_m = 12,290 \times 10^{-6}$ c.g.s.u. at 25°C ., corresponding to $\mu = 5.43$ Bohr magnetons (112)] had been established, it proved convenient in hemoglobin studies to determine both concentration and relative susceptibility by a utilization of these "standards" (104, 112). The working equation is

$$\chi_m = \frac{\Delta w - \Delta w_{\text{COHb}}}{\Delta w_{\text{Hb}} - \Delta w_{\text{COHb}}} \times 12,290 \times 10^{-6} \text{ c.g.s.u.}, \quad (\text{III})$$

where Δw , Δw_{Hb} , and Δw_{COHb} are respectively the forces measured (in milligrams) for the solution under investigation, for ferrohemoglobin, and for carbonmonoxyhemoglobin. The determined value of χ_m may be substituted in equation I for the calculation of the magnetic moment.

(ii) The susceptibility tube is suspended below a wooden table on which the balance is supported. The suspending connection between tube and one balance arm is a silk thread which passes through a hole in the tabletop and through the floor of the balance (one pan and sup-

port having been removed). Strong, constant magnetic fields (8830 gauss at 14 amperes) are applied from a half-ring, water-cooled electromagnet.

(iii) Under the experimental conditions used by Pauling *et al.*, Δw values as high as 50 mg. are obtained. To attain such readily measurable values, the present technique requires large amounts of material—one of the drawbacks of the method. The solutions used in the hemoglobin studies varied from 0.011 to 0.018 *M* upon an iron basis (104). These are very high concentrations, being approximately two hundred-fold greater than that used in spectrophotometric studies with 1 cm. cuvettes. In Theorell's magnetic measurements upon cytochrome-*c* (126) somewhat lower concentrations were employed, namely, 0.005 *M*. However, in this case also the method was wasteful of material, 2.45 gm. of ferricytochrome-*c* being required for each titration. This necessitated the use of impure preparations.

Results.—The accompanying table summarizes the general conclusions as to chemical bonding in the various iron porphyrin complexes studied by the magnetometric method.

The values of the effective moment per hemin unit calculated from the observed values of χ_m usually agree well with theory (equation II). However, in certain instances deviations from predicted values are found, as in the cases of ferro- and ferrihemoglobin. Here the deviations are ascribed to heme-heme interaction tending to stabilize configurations in which the moments of the four individual prosthetic groups are opposed (104). Such interactions must be very weak and cannot be shown magnetometrically to operate in the stepwise oxygenation equilibrium (146). Magnetic measurements indicate that the hemin units are essentially independent.

Deductions from spectrophotometric data have been found to closely parallel deductions from magnetometric measurements in the study of certain equilibria as well as of structure. The correlation of measurements by different techniques is brought out beautifully in the work of Theorell (124, 125, 126). The concordance of results in the study of the ferrihemoglobin-ferrihemoglobin hydroxide equilibrium (98, 103, 104) by different methods has already been pointed out.¹⁰ The writer independently has deduced (58, 149) from an analysis of their spectra the similarity of structure of ferrihemoglobin cyanide and the ferricyanide ion (Class II, Type B, see Table I). It must not

¹⁰ Cf. p. 548.

TABLE I

BOND TYPES IN IRON PORPHYRIN COMPLEXES

Derivative	Type	Reference
Class I, Type A (five unpaired electrons per iron atom)		
Ferriprotoporphyrin	Ionic	(141)
Ferrihemoglobin III (pK_3)	Ionic	(104)
Ferrihemoglobin I (pK_1)	Ionic	(102, 104)
Ferrihemoglobin fluoride	Ionic	(104)
Ethanol ferrihemoglobin*	Ionic	(145)
Ethanol ferrihemoglobin hydroxide†	Ionic	(145)
Ferricytochrome- <i>c</i> I and II	Ionic	(126)
Ferricytochrome- <i>c</i> fluoride	Ionic	(126)
Class I, Type C (four unpaired electrons per iron atom)		
Ferroprotoporphyrin	Ionic	(141)
Ferrohemoglobin	Ionic	(112, 144)
Class II, Type B (one unpaired electron per iron atom)		
Nitric oxide hemoglobin (ferro)‡	d^2sp^3 covalent	(146)
Ferrihemoglobin cyanide	d^2sp^3 covalent	(104)
Ferrihemoglobin hydrosulfide	d^2sp^3 covalent	(104)
Ferrihemoglobin azide	d^2sp^3 covalent	(145, 147)
Ammonia ferrihemoglobin hydroxide†	d^2sp^3 covalent	(145)
Imidazole ferrihemoglobin*	d^2sp^3 covalent	(148)
Ferricytochrome- <i>c</i> , III, IV, and V	d^2sp^3 covalent	(126)
Ferricytochrome- <i>c</i> cyanide	d^2sp^3 covalent	(126)
Class II, Type D (no unpaired electrons, diamagnetic)		
Oxyhemoglobin§	d^2sp^3 covalent	(144)
Carbonmonoxyhemoglobin§	d^2sp^3 covalent	(144)
Ethylisocyanide hemoglobin (ferro)*	d^2sp^3 covalent	(148)
Ferrocyclochrome- <i>c</i>	d^2sp^3 covalent	(126)
Pyridine ferroprotoporphyrin	d^2sp^3 covalent	(141)
Globin ferroprotoporphyrin	d^2sp^3 covalent	(141)
Class of Intermediate Types (three unpaired electrons per iron atom)		
Ferrihemoglobin hydroxide (pK_3)	Uncertain	(104, 145)

* Unique examples of complexes between ferrihemoglobin and a neutral electron donor.

† Structure not fully clear, but a large change in paramagnetism occurs upon addition of either alcohol or ammonia to ferrihemoglobin hydroxide, without evidence of displacement of hydroxide. The possibility of hepta-coordination is suggested. Cf. Clark *et al.* (3, 5), p. 546. The other complexes are all of the hexa-coordination type.

‡ The single electron is attributed to the nitric oxide group.

§ The iron oxygen and iron carbon bonds are considered to have partial double bond character. Cf. p. 548.

be assumed, however, that the different methods are solely of complementary value. Each has its special fields of usefulness as Clark *et al.* have shown (1 to 5) in the study of coordination equilibria in metalloporphyrin derivatives by means of electrometric and spectrophotometric techniques. In no other biological field have different physical methods been applied as extensively as in the study of hemoglobin and related compounds, and perhaps in no other field have the results of the newer techniques been as gratifying. It is hoped that the text of this review has served to emphasize the usefulness of the quantitative physical approach.

MISCELLANEOUS

Bibliographic references are given for a number of contributions of interest which have not been cited specifically in the text (150 to 156). These include papers on erythrocytins (150), hemerythrin (151), hemocyanins (152, 153), leucopterin (154), ferritin (a ferric nucleoprotein of very high iron content from spleen) (155), and urechrome, a new pigment from the worm, *Urechis caupo* (156).

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THE ALKALOIDS

BY LYMAN C. CRAIG

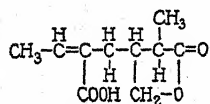
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The subject matter of this review and the manner of treating it will follow as nearly as possible the precedent set by the earlier reviews of the field. Where active work on some of the older topics has nearly ceased, topics less well understood and under development have been substituted.

One of the most helpful contributions to the entire field has been the appearance of the third edition of *The Plant Alkaloids* by T. A. Henry. A very ready background for anything discussed in the present review can easily be obtained by reference to this most excellent volume.

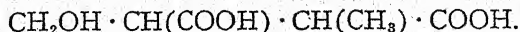
Senecio alkaloids.—Investigation of *Senecio* species heretofore not studied has continued to reveal the presence of senecionine (1). This substance appears to be the most widely occurring alkaloid of all the *Senecio* group.

Aside from reporting new members to this rapidly growing family Manske (1) offers structural formula I for senecic acid, the acid fragment of senecionine. The formula is based on the quite probable as-



I. Senecic acid

sumption of terpene character together with other observations which include the following considerations. Two carboxyl groups are present and a hydroxyl group is in such a position that it lactonizes with only one of the carboxyls. A single double bond is present and is so situated that acetic acid is produced on oxidation with permanganate. At least two C-methyl groups are present. The nitric acid oxidation product, $\text{C}_8\text{H}_8\text{O}_4$, of Barger & Blackie (2) seems most likely to be the lactone of a methylparaconic acid,



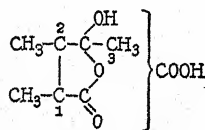
The different *Senecio* alkaloids are for the most part esters of a single alkaline, retronecine. However, there is found in the different members an almost bewildering diversity of structure in the acid portion of the different members. These acids are considered to be derivatives of terpenes (1, 2).

Men'shikov (3) has found the acidic fragment of heliotrine to be 2-methyl-3-hydroxy-4-methoxypentane-3-carboxylic acid.

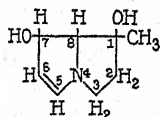
In the investigation of the toxic principle present in certain species of *Crotalaria*, Neal, Rusoff & Ahmann (4) reported the isolation of a new alkaloid which they called monocrotaline. Later Adams & Rogers (5) took up the study of this alkaloid and revised the original formulation to that of $C_{16}H_{23}O_6N$. Alkaline hydrolysis gave an acid, $C_7H_{12}O_6$, which was called monocrotic acid, and a base which proved to be identical with retronecine (87). Reductive cleavage of the alkaloid gave a different acid than did alkaline hydrolysis. This acid, $C_8H_{12}O_5$, was called monocrotalic acid. It readily lost carbon dioxide when treated with alkali and was converted into monocrotic acid.

Monocrotic acid yielded meso and racemic α, α' -dimethylsuccinic acid (6) on oxidation. It contained a carboxyl group and dehydrated on heating to a lactone which behaved as a β, γ -unsaturated lactone. α, β -dimethyllevulinic acid, the structure most likely, was then synthesized and found to be identical (7) with monocrotic acid.

Monocrotalic acid behaved as a lactone acid. Its structure was concluded to be that shown in formula II with the carboxyl located at either position 1, 2, or 3.



II. Monocrotalic acid



III. Retronecine

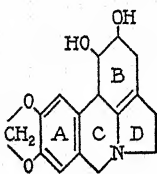
Adams & Rogers (8) have confirmed the 1-methylpyrrolidizine formula proposed by Men'shikov (87) for heliotridane and offer formula III for retronecine (8). The reasons for placing the double bond and the hydroxyls at the positions indicated are based on the following considerations: (a) One hydroxyl probably is tertiary since it is difficult to esterify in both retronecine and its dihydro derivative; (b) one hydroxyl undergoes hydrogenolysis readily and in this be-

havior is suggestive of a β,γ -unsaturated alcohol; (c) retronecine does not react with ketone reagents; (d) dihydroretronecine [found to be identical with platynecine obtained from hydrolysis of platyphylline, the alkaloid from *Senecio platyphyllus* (9)] loses water readily to form an anhydro derivative.

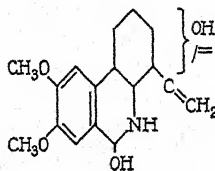
Aporphine group.—The three alkaloids isolated from *Artabotrys suaveolens* have been found by Barger & Sargent (10) to belong to the aporphine group. Artabotrine is probably 10-hydroxy-4,5,6-trimethoxy aporphine, while suaveoline appears to be 4,10-dihydro-5,6-dimethoxy aporphine. Artabotrinine is probably 2-methoxy-5,6-methylenedioxy aporphine.

The alkaloid anonaine, from *Anona reticulata* L., has been shown by Barger & Weitnauer (11) to be 5,6-methylenedioxy aporphine. Identity was established through synthesis of the racemic alkaloid and comparison of the Hofmann degradation product of the synthetic substance with that from the naturally occurring alkaloid.

Lycoris alkaloids.—Recent work by Kondo and his collaborators has cleared up many uncertainties in this field and has enabled the Japanese workers to offer a structural formula (12) for the main alkaloid (lycorine) of this interesting group which is in harmony with all the observations made thus far. The structural formula they offer is that shown in formula IV.



IV. Lycorine



V. Lycorenine

In the last review covering this field (87), evidence leading to the ring structure above, as well as to the position of the methylene dioxy group, was discussed. Two hydroxyls and the position of the double bond remained to be accounted for. The positions these could occupy have since been limited by the observation (12) that aromatization of ring B took place simultaneously with the Hofmann degradation and with the loss of the two hydroxyl groups. Ring B therefore probably contains these groups. Dihydrolycorine failed to undergo the Hofmann degradation and gave only starting material again.

Dihydrolycorine was found to react with lead tetraacetate to yield a substance which would not itself crystallize but gave a crystalline dioxime (12). The two hydroxyl groups thus appear to be vicinal to each other. They can be acetylated readily and apparently are of secondary character.

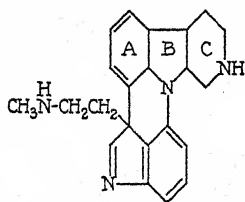
The positions of the double bond and hydroxyl groups could now be deduced from absorption spectra data (12) together with the above information. The absorption spectra of lycorine itself proved to be almost the same as that found for the dihydro derivative, which indicated that the double bond is not conjugated with the aromatic ring. The most likely positions for the two hydroxyl groups and the double bond, barring rearrangements, are those given in formula IV. The absorption spectrum of the anhydromethine derivative obtained through the Emde degradation indicated conjugation of the double bonds with the aromatic nucleus.

Kondo & Ikeda (13) have also obtained considerable evidence regarding the nature of another *Lycoris* alkaloid, lycorenine, for which the empirical formula $C_{18}H_{23}O_4N$ was given. This substance contains two methoxyl groups, one methylimide group, and furnishes a mono- as well as a diacetyl derivative. By catalytic hydrogenation a dihydro derivative was obtained. A two-step Hofmann degradation removed the nitrogen as trimethylamine and gave a crystalline compound, $C_{15}H_{10}O(OCH_3)_2$, which reacted with hydroxylamine to give an oxime. The des-N-compound reacted with ozone to give formaldehyde, a dialdehyde, and an acidic aldehyde. The latter upon oxidation with potassium permanganate gave a dibasic acid. The identity of this acid was established through the synthesis of its ester as 3,4-dimethoxybiphenyl-6,3'-dicarboxylic acid. Two other alkaloids of *Lycoris* have previously been shown to be derived from a phenanthridine nucleus and the above observation can readily be harmonized with the structures previously proposed by means of formula V. Ozone upon lycorenine itself gave formaldehyde which indicates the presence of a vinyl side chain. Furthermore, hydroxylamine gave an oxime with lycorenine in a manner similar to cotarnine.¹

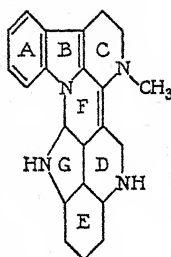
Calycanthine.—The formulation of this unique alkaloid first isolated by Eccles (14) in 1888 from *Calycanthus glaucus* has been changed to that of $C_{22}H_{26}N_4$ by Barger, Madinaveitia & Strueli (15).

¹ Compare the behavior of pseudostrychnine with hydroxylamine, page 586 of this review.

Heating this base with soda lime has given a high yield of methyltryptamine. The benzoyl derivative of this product was also formed by oxidative degradation of benzoylcalycanthine (16). Marion & Manske (17) obtained quinoline and 4-carboline as degradation products. The action of soda lime on benzoylcalycanthine gave 2-phenylindole (15). Largely on the basis of biogenetic possibilities involving the condensation of two tryptophane residues Barger, Madinaveitia & Strueli have proposed formula VI as a possible structural formula for calycanthine. A variety of reagents were found to be capable of trans-



VI. Calycanthine (formula of Barger *et al.*)



VII. Calycanthine (formula of Manske & Marion)

forming calycanthine into a base believed to have the formulation $C_{16}H_{10}N_2$, which was called calycanine. This latter base was considered to be formed by elimination of the side chain attached to the quaternary carbon atom and elimination of ring C.

Manske & Marion (18) have presented certain evidence not in accord with such a diindolylen structural formula and have suggested for calycanthine a structure, formula VII, which involves only one tryptophane residue. This formula was suggested largely by the selenium dehydrogenation products of calycanthine which include norharman, 4-methylquinoline, skatole, β -ethylindole, and calycanine. Evidence for rings F and G is less direct than for the others but the presence of the former may be readily inferred from the degradation products and certain other properties of the alkaloid. The fourth nitrogen is not directly located by the degradation products but is placed so that it completes ring G in order to account for the rather low temperature required to split out ammonia upon treatment with palladium and to account for the ease with which the N-C bond of the indole nitrogen is ruptured upon benzoylation (18). This particular

position for the nitrogen might also explain why nitrogen is so easily eliminated as methylamine in simple methylation experiments with methyl iodide.

The base calycanine could be formed by loss of the nitrogen of ring G and of the methyl attached to the nitrogen of ring C together with aromatization of rings C, D, and E (or possibly additional rupture of the N-C bond of the indole nitrogen). The analytical data for calycanine would be in agreement also with such a formula, $C_{21}H_{15}N_3$, as well as for the one earlier proposed.

The formula of Manske & Marion appears quite probable but will naturally require further substantiation. It does not give a ready explanation of the formation of 2-phenylindole on heating benzoylcalycanthine with soda lime.

Curare.—Since the last report (87) the careful work of Wieland and co-workers has added information to the problem of calabash curare. Thus far ten different alkaloids have been isolated (19) and these are believed to constitute only a portion of the different alkaloids actually present in the complex mixture. All were not found in every sample examined and indeed some could only be isolated from material having its source in certain regions. All the evidence seems to indicate that an unknown number of different species of plants has been used for the collection of calabash curare.

Some support for the original assumption (87) that *Strychnos toxifera* is a constituent of C-curare now appears to be offered by the isolation of an alkaloid (toxiferine II) from the mixture which is also present in identified specimens of *Strychnos toxifera*.

All of the alkaloids thus far isolated are of a type $C_{20}H_{25}N_2^+$ with more or less of the elements of water and hydrogen combined. This type of alkaloid is therefore characteristic of C-curare. Separation and isolation have been accomplished through careful and ingenious manipulation of their salts, such as chromatographic separation of Reinecke salts.

A résumé of this work can best be seen from Table I, which tabulates the alkaloids together with their formulas, rotations, and the melting points of the picrates. They show considerable variation in toxicity to the frog.

Very little is known about the chemistry of these substances. They must have at least one aromatic ring to account for their behavior (20) upon bromination and nitration. The hydrochloride of one of the alkaloids, C-curarine I, when treated with silver oxide to remove the

halogen apparently gives first the expected quaternary base and then loses water to give a tertiary base which is formed by the condensation of two molecules of the original substance (21). Quinolinium bases are known to undergo such a change (22) and it is logical to assume that these alkaloids may therefore contain a quinoline or isoquinoline ring system.

TABLE I
CURARE ALKALOIDS THUS FAR ISOLATED

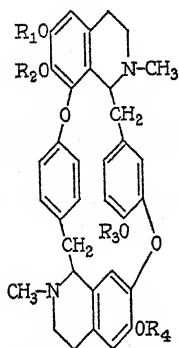
Name	Formula	Physiological activity*	Rotation	M.p. of picrate
C-Curarine I	$C_{20}H_{21}N_2^+$	3 to 4	+70 to 73°	>300°
C-Curarine III	$C_{20}H_{21}N_2^+$	500		
Toxiferine I	$C_{20}H_{21}N_2^+ \cdot (H_2O)$	inactive	—930°	189°
C-Curarine II	$C_{20}H_{23}N_2^+ \cdot (H_2O)$	0.3	—610°	270°
C-Dihydrotoxiferine I..	$C_{20}H_{23}N_2^+ \cdot (H_2O)$	50 to 100	+72 to 74°	204°
C-Isodihydrotoxiferine I	$C_{20}H_{23}N_2^+$	1.5	—605°	185°
Toxiferine II	$C_{20}H_{23}N_2^+$	3 to 4	—550°	242°
Toxiferine IIa	$C_{20}H_{23}N_2^+ \cdot (H_2O)$	5	216°
Toxiferine IIb	$C_{20}H_{23}N_2^+ \cdot (H_2O)$	20 to 30	+66 to 67°	210°
C-Toxiferine II	$C_{20}H_{23}N_2^+ \cdot (H_2O)$	100 to 150	+78°	216°
	$C_{20}H_{23}N_2^+ \cdot (H_2O)$	10	+72°	215°

* Wt. in μ g. of minimum lethal frog dose.

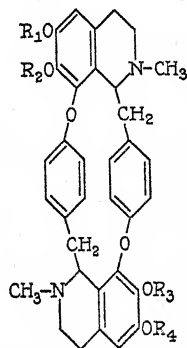
Further confirmation and enlargement of the information concerning the alkaloids present in tube and pot curare has been offered by King (23). A number of these alkaloids may now be classified according to two general types. One type, the isochondrodendrine type, which includes *d*-isochondrodendrine, *O*-methylisochondrodendrine, *d*-protocuridine, and *i*-neoprotocuridine, may be represented by formula VIII. The other, the bebeerine type, which includes *d*-bebeerine, *l*-bebeerine (*l*-curine), *l*-chondrofoline, and *d*-tubocurarine, may be represented by formula IX. Chondrofoline is a new alkaloid isolated from the leaves of *Ch. platyphyllum*.

King also reported an interesting observation on the botanical origin of the substance known in pharmacy as "Radix pareira bravae," which has long been a matter of some dispute. When *l*-bebeerine could be isolated from the substance, it indicated that *Ch. platyphyllum* was the botanical source, but when *d*-bebeerine was found *Ch. microphyllum* was indicated as the more probable botanical source.

Erythrina.—An extensive investigation of various species of *Erythrina* for substances with curare-like action is being carried out by Folkers, Koniuszy and collaborators. Although as early as 1888 the curare-like action of extracts of the seeds of *Erythrina americana*



VIII. Isochondrodendrine type
of curare alkaloids



IX. Bebeerine type of
curare alkaloids

Mill was known (24), a successful attempt to isolate a definite substance responsible for the action was not made until Folkers & Major (25) reported the isolation of the new alkaloid erythroidine. This substance possessed curare-like action and moreover, in contrast to curare, was active when administered orally.

The therapeutic possibilities then stimulated a thorough investigation of extracts from different species of *Erythrina*. Thus far 51 of the 105 known species have been investigated (26) and all have been found to have activity in varying degree. Alkaloids which paralyze the nerve endings of voluntary muscles have in the past been found in plants from the genus *Strychnos* as well as from those in the Menispermaceae and Capparidaceae. The genus *Erythrina* now definitely must be included in the list.

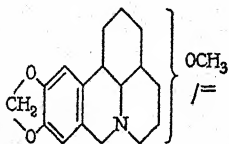
Thus far a series of ten different alkaloids whose structure is probably closely related have been isolated. They are alpha and beta erythroidine, $C_{16}H_{18}NO_3$ (27), erythramine, $C_{18}H_{21}NO_3$ (28a), erythraline, $C_{18}H_{19}NO_3$, erythratine, $C_{18}H_{21}NO_4$ (28b), erysodine, $C_{18}H_{21}NO_3$, erysopine, $C_{17}H_{19}NO_3$, erysocine, $C_{18}H_{21}NO_3$, erysovine, $C_{18}H_{21}NO_3$ (28c), and erysonine, $C_{17}H_{19}NO_3$ (28d).

Erythramine (28e) and erythraline (28f) have been examined from the standpoint of constitution and may be considered representa-

tive of the others. The former contains a single ethylenic linkage as contrasted to the latter which has two. This appears to be their only difference since the hydrogenation products proved to be identical. Both possess a methylene dioxy group, a methoxyl group and a tertiary nitrogen which is probably common to two rings since alpha and beta erythroidine (27) required a three-stage Hofmann degradation for the elimination of the nitrogen and since their tertiary nitrogen did not become of secondary character upon hydrogenation. The Kuhn-Roth determination for C-methyl was negative.

Oxidation of erythraline methohydroxide with potassium permanganate gave an orthodicarboxylic acid which was isolated as its methylimide derivative. This latter substance proved to be identical with hydrastic acid methylimide. The presence of a tetrahydroisoquinoline ring system was thus suggested and was further supported by good agreement in the absorption spectrum of dihydroerythramine with that of 6,7-methylenedioxy-1,2,3,4-tetrahydroisoquinoline.

Two additional rings must be present which are hydroaromatic and should prove to be condensed with the heterocyclic ring of the isoquinoline nucleus because of the limitations of the formulation and of the preceding observations. A number of arrangements are possible. One of these might be the structure now established for the alkaloid lycorine (page 571 of this review). A relationship to tazettine (29) is also not excluded. A possible structure unique in certain respects but preferred by the authors is that shown in formula X.

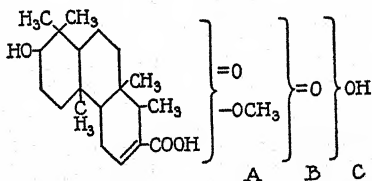


X. Erythramine

The erythrophleum alkaloids.—For a long time certain members of the genus *Erythrophleum* have been known to contain a toxic principle. The first alkaloid isolated from a plant of this genus was called erythrophleine by Gallois & Hardy (30). This highly toxic substance appeared to be unique in that it exhibited a type of physiological action characteristic of the cardiac glycosides and also possessed local anesthetic properties. In more recent years Dalma has taken up the study of the toxic principle and in collaboration with Ruzicka now reports

the isolation of a number of crystalline alkaloids, among which may be mentioned cassaine, $C_{24}H_{39}O_4N$ (31), cassaidine, $C_{24}H_{41}O_4N$ (32), and coumagine, $C_{28}H_{45}O_6N$ (33).

Cassaine was found to give a mono-oxime and a monoacetyl derivative (31). Hydrolysis with dilute acid gave a base which proved to be dimethylaminoethanol (34) and an acid, $C_{20}H_{31}O_4$ (31), which was called cassianic acid. A single double bond was present in both the alkaloid and the acid. Absorption spectrum studies indicated the double bond to occupy a position alpha-beta to the carboxyl group (32). Dehydrogenation with selenium gave 1,7,8-trimethylphenanthrene. Blount, Openshaw & Todd (35) hydrolyzed the amorphous erythrophleine and obtained a crystalline acid which upon dehydrogenation also gave 1,7,8-trimethylphenanthrene. On the basis of the trimethylphenanthrene as well as certain other considerations the acid may be diterpenoid in character and the tentative structures shown in formula XI may be suggested (32, 35) for the acidic portion



XI. A = Erythrophleic acid
 B = Cassianic acid
 C = Cassaidinic acid

of the alkaloids, although other possibilities are not yet excluded. The alkaloids themselves are the dimethylaminoethanol esters of these structures.

Colchicine.—Cohen, Cook & Roe (36) have drawn attention to several respects in which the Windaus (37) formula for colchicine (87) seems unsatisfactory. Colchicol methyl ether as represented by the Windaus formula should be a 9,10-dihydrophenanthrene derivative and thus should pass easily into the completely aromatic state. However, it fails to do this as does the corresponding carbinol obtained by replacement of the amino group by hydroxyl. However, the absorption spectrum of acetyl colchicol methyl ether is compatible with the 9,10-dihydrophenanthrene formula, whereas the corresponding carbinol shows considerable shift in the position of the absorption

bands and may possibly be formed by a rearrangement. A formula more in agreement with the experimental observations than the Windaus formula has not as yet been offered.

Solanum alkaloids.—In recent years a certain amount of simplification has been brought about in this rather confusing field and the formulations are on more certain ground. For the most part the alkaloidal moieties occur in the plant attached to sugars in glucosidal union and therefore may be considered as aglucones. Where this is the case much uncertainty still persists as to the identity, number of sugar fragments, the order of attachment, etc.

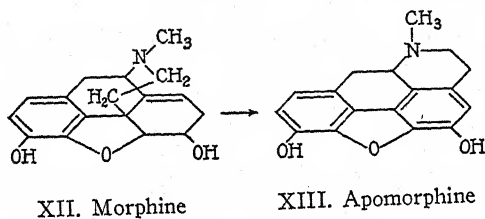
For purposes of structural studies the following alkaloids (aglucones) certainly must be taken into account: solanidine, $C_{27}H_{43}NO$ (38), solasodine, $C_{27}H_{43}NO_2$ (39, 40), solangustidine, $C_{27}H_{43}NO_2$ (41), solanocapsine, $C_{26}H_{44}NO_2$, and solanocapsidine, $C_{26}H_{44}N_2O_4$ (42). The solaneine isolated by Firbas (43) has been shown by Soltys (44) to be a mixture of solanine and solanidine. Solanthrene is solanidine in which the hydroxyl has been lost with the formation of a double bond (45). Likewise solanosodine is solasodine (40) in which the hydroxyl of carbon atom 3 has been lost. Rochelmeyer (40) and Briggs (39) have found the solankarpidine of Sayed & Kanga (46) to be identical with solasodine.

Three of the five alkaloids named above have been found upon dehydrogenation with selenium to give Diels hydrocarbon and there seems little doubt that they are sterol derivatives. Furthermore, the first two are precipitated by digitonin and the transformations which involve one of the hydroxyl groups as well as the double bond in solanidine and solasidine are quite parallel with those which concern the hydroxyl group on carbon atom 3 of the sterols and the double bond when it is located in the 5,6 position (40). The nature of the nitrogen heterocyclic portion of the molecule is still unsettled, although it is undoubtedly made up of two five- or six-membered rings which have the nitrogen common to both rings, and it is presumably molded from the sterol side chain by condensation with one nitrogen atom. The nitrogen does not undergo the Hofmann degradation readily. Some indication of the formation of both pyrroles and pyridines in pyrrolytic degradations has been reported.

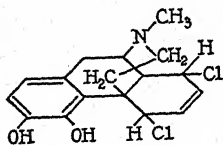
The opium alkaloids.—A considerable amount of excellent synthetic work in this field has been reported by Small and co-workers in the past few years. This work has permitted enlargement of our knowledge regarding the effect that various substituents have on the

physiological action. The collected reprints of these extensive studies may now be found in "The National Research Council Report of the Committee on Drug Addiction 1929-1941." In these studies nothing apparently was found which would cast doubt on the fairly well established structural formula of morphine.

A new and logical explanation of the morphine-apomorphine transformation has been offered by Small, Faris & Mallonee (47). When morphine is treated with hydrochloric acid at 140° (48), a change in structure from formula XII to formula XIII takes place.



For a long time the way in which this transformation occurred remained obscure, but was explained on the basis of a rearrangement by Schöpf & Hirsch (49). Small, Faris & Mallonee more recently have found the dichlorodihydrodesoxymorphine (formula XIV) to be an intermediate in the transformation and a more simple mecha-



XIV. Dichlorodihydrodesoxymorphine

nism than the earlier one is at once apparent. The dichloro derivative is formed by the action of hydrochloric acid on morphine and probably loses HCl at the 8,14 positions, after which there is an alpha-gamma shift of the chain attached to carbon atom 13 to position 8. Coincidental with this a further molecule of HCl is lost with the formation of a benzene ring to give apomorphine, formula XIII.

Aconite alkaloids.—Progress in this difficult field has been slow but has led to a better understanding of the nature of the oxidation of aconitine and derivatives (50). These transformations appear to be concerned mostly with the periphery of the molecule and do not

represent extensive degradation. Because the more complex alkaloids do not lend themselves to ready degradation, some attention has been directed to certain members of the group which contain less oxygen. Of significance for this approach is the study of atisine (51), napelline (52), the new alkaloid from *Delphinium staphisagaria*, staphisine, described by Jacobs & Craig (53), and the new alkaloid of Sugimoto & Shimanouti (54), kobusin, from the mother liquors of jesaconitine.

There is no doubt now that the delphinium alkaloids belong to the aconite group, for the parallelism in the transformations of aconitine and delphinine is quite striking (55). Delphinine was found to contain an acetic ester grouping as well as the benzoic ester grouping earlier reported (56). The formulation of delphinine has been revised to $C_{33}H_{45}NO_9$.

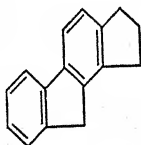
Selenium dehydrogenation of staphisine (53) has led to a series of hydrocarbons which are of obvious homologous character and have the properties of alkyl phenanthrenes. The similarity of their absorption spectra with that of phenanthrene leaves little doubt as to their ring structure. Atisine was found by Lawson & Topps (51) to give a hydrocarbon on dehydrogenation, $C_{17}H_{16}$, which was believed to be a phenanthrene; napelline was found by Rogers & Freudenberg (52) to behave similarly. More recent comparison of these two hydrocarbons has shown them not to be identical, although their picrates and trinitrobenzene derivatives are similar (57). A $C_{17}H_{16}$ hydrocarbon was not found among the dehydrogenation products of staphisine. It seems entirely probable now that the simpler types of aconite alkaloid contain a hydrogenated phenanthrene nucleus, but the fact that they apparently do not give the same phenanthrene hydrocarbons on dehydrogenation makes their exact relationship very difficult indeed to derive experimentally.

No suspicion of the nature of the nitrogen heterocyclic portion of the molecule has as yet been offered. The data at hand on the formulations of staphisine and of napelline as well as of the new alkaloid kobusin (54) indicate them to be hexacyclic. Three rings, the nature of which cannot as yet be surmised, thus remain to be accounted for. At present, on the basis of the published data of Lawson & Topps alone, it is difficult to see the manner in which atisine fits into the aconite picture, since such data would indicate it to be pentacyclic in nature (87).

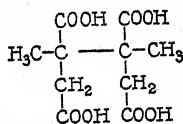
It seems probable that the more highly hydroxylated aconite alkaloids also contain a hydrogenated phenanthrene ring system, although

thus far no definite crystalline hydrocarbon has been reported from their dehydrogenation.

Veratrine group.—The number of degradation products characteristic of cevine, the alkamine of veratrine, has been enlarged considerably by Jacobs & Craig since the last report (87) and offer pertinent suggestions regarding the long obscure structure of this substance. Five hydrocarbons have been isolated from the selenium dehydrogenation of cevine (58). Four of these apparently are homologous and are postulated to be cyclopentenofluorene (58) derivatives (formula XV) on the basis of absorption spectra data as well as from other considerations. This ring structure, if present in hydrogenated



XV. Cyclopentenofluorene



XVI

form in the original alkaloid, would account for four of the six rings indicated by the empirical formula (58). The remaining two may be accounted for by the heterocyclic portion of the molecule which contains a nitrogen atom common to two rings (59). One of these rings most certainly is six-membered, since an optically active piperidine derivative has been obtained by soda-lime distillation (59) and also a number of other degradation products which are pyridine derivatives. The other ring is either five- or six-membered. The basic portion of the molecule is therefore most likely an octahydropyridocoline or pyrrocoline derivative, but direct evidence for the position of attachment of this system to that of the nonbasic portion of the molecule has not yet been derived.

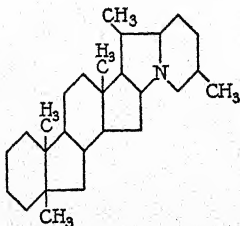
The formulation of the dehydrogenation product of cevanthridine (88) has been revised to that of $C_{25}H_{27}N$ (58). Upon hydrogenation, this substance forms a tetrahydro derivative which contains a secondary nitrogen atom and can scarcely be a phenanthridine derivative as previously supposed (88). Its relationship to the hydrocarbons mentioned above was shown by the striking similarity of the absorption spectra of the tetrahydro derivative and of the hydrocarbons. Cevanthridine must therefore contain the same ring system and in addition a six-membered nitrogen heterocyclic ring condensed at some point

with one of the benzene rings. Furthermore, from the absorption spectrum measurements it is indicated that the nitrogen of this ring is probably removed by at least one carbon atom from the aromatic ring in the tetrahydro derivative and thus in cevanthridine itself. This observation would indicate cevanthridine to be an isoquinoline derivative.

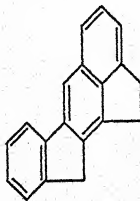
An interesting series of polycarboxylic acids was isolated from the chromic acid oxidation of cevine by fractional distillation of their methyl esters (60). The latter were found to contain labile ester groups as well as those resistant to hydrolysis by alkali and therefore of tertiary character. One of these substances, a hexanetetracarboxylic acid, was concluded to possess formula XVI, since it formed a dianhydride on distillation under high vacuum, a ketoanhydride on redistillation at a higher temperature, was optically active (as well as the two anhydrides), and contained two tertiary carboxyl groups. If the correctness of this formula is assumed, then two geminal methyl groups must be present in cevine attached to adjacent carbon atoms.

Another acid isolated as the trimethyl ester, $C_{17}H_{24}O_8$, gave on hydrolysis and subsequent heating a crystalline lactonic acid, $C_{14}H_{14}O_6$, which was called "decevinic acid." The latter had been previously isolated directly from the oxidation products after preliminary heating (60). The exact nature of this substance, apparently formed by rearrangement during the heating, has not as yet been satisfactorily explained.

Although more than thirty degradation products of widely differing character have been isolated from cevine, they do not as yet indicate with assurance a complete structural formula for the ring system. The degradation products thus far encountered, however, might all originate from a ring system such as that shown in formula XVII. Several features of this formula are not directly indicated by the



XVII. Possible ring system of cevine

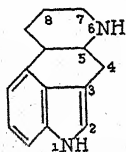


XVIII. Ring system of the benzocyclopentenofluorenes

degradation products but are only considered on the basis of certain relationships known to exist in hydroaromatic natural products of known structure.

Jacobs & Craig have subjected jervine to selenium dehydrogenation (61) and have isolated a series of hydrocarbons definitely of the same type as those which came from cevine. In addition, a second series of hydrocarbons were isolated which contained a further aromatic six-membered ring and were interpreted as being benzocyclopentenofluorenes, formula XVIII. The results obtained with jervine strengthen considerably the view taken regarding the nature of the hydrocarbons from the dehydrogenation of cevine. Basic fragments also were formed and proved to contain the 2-ethyl-5-methylpyridine previously isolated from the selenium dehydrogenation of cevine (58). A close relationship evidently exists between the two types of veratrine alkaloids, although the jervine type contains a nitrogen which behaves as if it were secondary in character (62). The suggested presence of a methylene dioxy group has been disproved (63).

The alkaloids of ergot.—Further attempts by Jacobs & Gould to confirm by synthetic procedures the structural formula for lysergic acid (87), derived for the most part through degradation studies, have met with a measure of success. The base ergoline (64), formula XIX, was synthesized and found to give the color tests characteristic of the ergot alkaloids with but slight difference. 6-Methylergoline was also synthesized (64) and found to give color tests very close to those shown by lysergic acid itself.



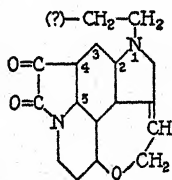
XIX. Ergoline

Synthesis of lysergic acid or of the dihydro derivative has offered a very difficult problem. However, a transformation product of dihydrolysergic acid, considered to be 8-methyl-7-oxo-6-methylergoline (64), has been hydrogenated to 6,8-dimethylergoline. This latter substance has also been synthesized. The product obtained from lysergic acid, however, was optically active (3 asymmetric carbon atoms) while the synthetic product was naturally racemic. Otherwise

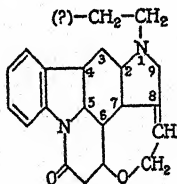
close agreement in the properties of the substances from both sources was demonstrated. The difficulty of optical activity has been partially overcome by the use of racemic lysergic acid (65) for the transformation to 6,8-dimethylergoline. Although the slight yield of the final product did not allow an entirely satisfactory comparison to be made (66), the melting point was now close to the synthetic material and also did not show a depression when a mixed melting point was taken. All things being considered, there appears to be little doubt as to the validity of the structures proposed.

Strychnine group.—Many papers in this field have appeared since the last review. They illuminate considerably the transformations which the numerous derivatives of this class of intricate substances can undergo.

Of these careful studies, first in importance from a structural standpoint might be mentioned the reinterpretation of the bromination studies (67) of diketonucidine originally made by Leuchs. On the basis of these earlier studies, diketonucidine (formula XX) was considered to give readily a monobromo compound; this would indicate the presence of a replaceable hydrogen on a carbon atom adjacent to a carbonyl group, viz. carbon atom 4. The extensive studies already



XX. Diketonucidine



XXI. Strychnine

treated in previous reviews appear to suggest rather clearly formula XXI for strychnine with uncertainty remaining with respect to the position of attachment of one end of the $-\text{CH}_2\text{CH}_2-$ group; only positions 3, 4, and 5 are possible if the other details of the formula are correct. The bromination studies mentioned above appeared to eliminate position 4 from consideration, although otherwise it seemed the more logical of the three. Certain objections presented themselves for both position 3 and 5. The structural formula has thus remained uncertain in regard to this point for a number of years.

Leuchs & Grunow (68) have recently repeated the original bromination study and although the earlier experimental data were con-

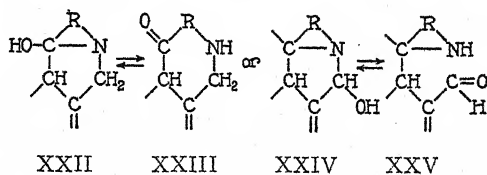
firmed the different conclusion has now been reached that bromine does not substitute in position 4 but rather a reaction comes about by addition of bromhydrin to the double bond of diketonucidine. Robinson & Holmes (69) have also repeated the original bromination study of Leuchs but were unable to isolate a simple bromination product which a grouping $-\text{COCOCH}-$ should afford if it were present. The English workers view the failure to brominate smoothly as favoring the absence of hydrogen on carbon atom 4, which may in turn be interpreted as indicating the attachment of the ethylene group at this position. Such an arrangement would also be more compatible with the appreciable yield of tryptamine obtained from strychnine on heating with alkali (70). Other evidence bearing on position 4 for the ethylene group has been fully discussed by Holmes & Robinson.

If formula XXI with the ethylene group joined at position 4 is accepted as the true formula for strychnine, then it contains an octahydropyrrocoline ring system and the transformations which are concerned with the basic nitrogen may be those characteristic of such a system. No doubt the vicinity of the double bond also plays an important role. In the case of strychnine and its derivatives the experimental data have been found to be of unusual complexity and often capable of more than one interpretation. The octahydropyrrocoline ring system has heretofore not been common in alkaloid chemistry but now, aside from strychnine, is found in the *Lycoris* alkaloids and perhaps in the veratrine alkaloids.

Strychnine can be readily oxidized to pseudostrychnine (71) by oxygen in the presence of the copper-ammonium complex. This oxidation product was also found in the mother liquors from the preparation of strychnine (72). Pseudostrychnine differs from strychnine by the addition of a single oxygen atom which is attached to a carbon atom contiguous to the basic nitrogen atom $\text{N}_{(b)}$ (73). It retains most of the original basicity of strychnine. The oxygen in question behaves as a hydroxyl in certain reactions and forms an ether when dissolved in hot alcoholic solution (71). Although the oxygen was originally considered to be joined to carbon atom 9, Robinson & Blount found it to resist oxidation to carbonyl (amide linkage) and suggested that it might better be considered as a tertiary hydroxyl rather than secondary. Evidence was thus offered that carbon atom 2 is the one in question.

From the first it was recognized that pseudostrychnine did not behave as a single substance (72, 73) but formed an N-acetyl derivative

with acetic anhydride rather than the expected O-acetyl derivative. It also gave an N-nitroso derivative. However, it failed to react with ketone reagents. On the basis of this and later work (74) it now seems clear that pseudostrychnine can show a change as follows (75) depending² on which position is accepted as the one for the oxygen.



The carbonyl group evidently enolizes readily to form the O-alkyl ethers.

The O-methyl derivative of pseudostrychnine, formed by dissolving the alkaloid in hot methyl alcohol, adds methyl iodide readily and two products can be isolated (75). One is the N-methyl methiodide of XXIII and the other is the hydriodide of a tertiary base isomeric with the original methoxy base. The latter is the hydriodide of the N-methyl derivative of XXIII. Both are explained on the basis of migration of methyl from oxygen to the nitrogen.

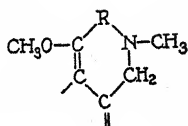
Pseudobrucine behaves somewhat more simply and gives directly, in good yield, the hydriodide of the N-methylated tertiary base. Either a rearrangement must take place after the addition of the secondary form must be present in the starting material.

When the quaternary derivative (methylmethiodide of XXIII) is treated with sodium methylate, a rearrangement of methyl back to oxygen takes place with the formation of XXVI. In the case of pseudostrychnine this O-methyl derivative will undergo hydrolysis of the methyl ether when treated with acid to give an N-methylpseudostrychnine derivative which is tertiary. In the case of pseudobrucine the methyl first wanders back to the nitrogen to form a quaternary salt and then can readily be split off as methyl iodide in the Zeisel determination.

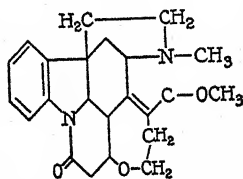
More recently some interesting information has been contributed by Leuchs (76) in his study of the acetylation of pseudobrucine and pseudostrychnine and the oxidation of the resulting derivatives with permanganate. When either base was subjected to acetylation with

² R is the substituted six-membered ring attached in these two positions.

acetic anhydride in pyridine at a temperature of 100° , a product was obtained which had added one acetyl group. This material no longer had the basic properties which the O-acetyl derivative of XXII would have. Therefore the acetylation must have taken place on the nitrogen and the N-acetyl derivative of XXIII is indicated. A similar result had previously been obtained in the acetylation of dihydropseudostrychnine (77).

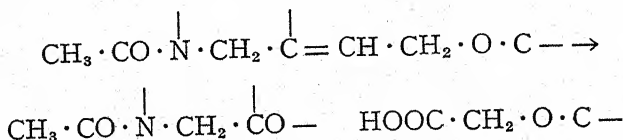


XXVI



XXVII. Methoxymethyldihydro-neostrychnine

Furthermore, the original choice between XXII and XXIV was now supported by the data obtained in the oxidation of the acetyl derivatives with permanganate in acetone. Three oxygen atoms were taken up and a ketonic acid resulted. Leuchs interprets this to indicate that the double bond in pseudostrychnine did not wander but remained in the same position that it occupied in the original alkaloid. He believes the oxidation to take place as follows:



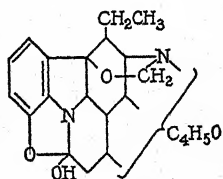
Such an interpretation is not unlikely if the hydroxyl of the pseudo series is located at position 2.

It is interesting to compare the earlier behavior of pseudostrychnine with certain transformations that take place with the metho salts of strychnine and derivatives. When these are treated with sodium methylate, the normal Hofmann degradation does not take place but instead a compound, methoxymethyldihydroneostrychnine, with formula XXVII is formed (78). In this formula the revised position for the ethylene group is used and the position of the double bond is that favored by Robinson and collaborators. That preferred by Leuchs and collaborators (79) would be the 2,7 position. On treatment with dilute

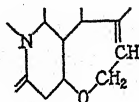
hydrochloric acid this base again reconstitutes a quaternary salt which differs from the original quaternary salt by the position of the double bond. It forms neostrychnine on distillation. The latter yields the same dihydro derivative on hydrogenation as strychnine itself. The re-forming of a quaternary salt is a unique reaction, for it appears to be a reversal of the Hofmann degradation. Moreover, in this reaction carbon atom 9 seems definitely to be the one involved if the interpretation proposed by Robinson and co-workers (80) for the transformations taking place when methoxymethyldihydroneostrychnine is subjected to oxidation by perbenzoic acid and subsequently reduced, is assumed to be correct.

The Hofmann degradation can theoretically take place also at either of the other carbon linkages on the basic nitrogen atom. Thus several different paths might be followed before the basic nitrogen is finally eliminated as trimethylamine and a very complex picture is presented. Recent work along this line is reported in a series of papers by Achmatowicz and co-workers (81).

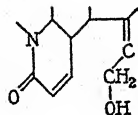
Vomicine.—In the last report (87) the studies of Wieland and co-workers which indicated the probable partial formula (XXVIII)



XXVIII. Vomicine, partial formula



XXIX



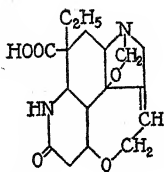
XXX

for vomicine were mentioned. This formula may now be extended and also revised in certain points.

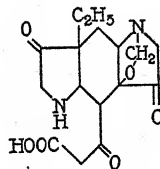
The analogy with the strychnine series would seem to favor position 4 (82, 86) for the ethyl group in view of the recent assignment of the 4 position for the $-\text{CH}_2\text{CH}_2-$ group in strychnine (68, 69). Furthermore, the parallelism found to exist with the strychnine series in the behavior toward hydrobromic and hydriodic acids and in the nature of the reduction products strongly suggests for vomicine as well the partial formula XXIX which had previously been established for strychnine (82). The change from vomicine to isovomicine (as well as of strychnine to isostrychnine) on treatment with hydro-

bromic acid may be represented by the change from formula XXIX to XXX.

The two acids, $C_{17}H_{22}N_2O_5$ and $C_{16}H_{21}N_2O_6$, obtained upon chromic acid oxidation of vomicine (83) are now considered to have formulas XXXI and XXXII respectively. Similar oxidation of dihydrovomycinine has given a substance, $C_{16}H_{20}N_2O_4$ (82), which has an

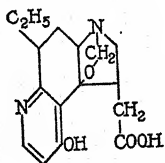


XXXI. Oxidation product of vomicine

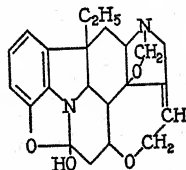


XXXII. Oxidation product of vomicine

aromatic ring and probably resulted from the dehydrogenation of the six-membered nitrogen ring as shown in formula XXXIII.



XXXIII. Oxidation product of dihydrovomycinine



XXXIV. Vomicine

If the pyridine ring can be formed as represented without disturbing the oxymethylene group, then a position on the nitrogen heterocyclic ring is excluded and position 7 is favored for the attachment of the oxygen of the oxymethylene group.

The foregoing partial structures when combined with formula XXVIII would lead to the probable formula XXXIV for vomicine. This formula should be accepted with the proper reservations since several of the features are only assumed and are based on analogy to strychnine.

The $=N-CH_2-O-$ group shown in this formula is of considerable interest, since it has not been encountered as yet in alkaloid chemistry. In the methylimide determination vomicine gives one NCH_3 (84) and therefore the methylene oxide ring in this case must

be split hydrolytically between the oxygen and the carbon adjoining the nitrogen. It appears to be split in this position also when the $C_{16}H_{24}N_2O_2$ base from the chromic acid oxidation of vomitidine is subjected to catalytic hydrogenation (85). On the other hand, when the quaternary salts of vomitine are subjected to the Emde degradation, the methylene oxide ring is split between the nitrogen and the carbon and a methoxyl-N-methyl derivative results.

The exact structure of vomipyrine, the product obtained from the dehydrogenation of the oxidation product of vomitidine, $C_{16}H_{24}N_2O_2$, has not yet been confirmed synthetically (86), although it seems most likely that it is a 5,6(N)-pyrroquinoline. On the basis of the revised structures presented above the 8,11-diethyl derivative might be expected; this was synthesized by Horner (86) but it did not prove to be identical with vomipyrine.

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MINERAL NUTRITION OF PLANTS

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INTRODUCTION

It has been well said recently by Seifriz (1) that "a scientist must look at his problem with the precise myopic eye of an experimenter, and also with the far seeing vision of the scholar." In considering current papers on mineral nutrition of plants one becomes impressed by the importance of the second part of this precept. In a number of cases there is insufficient application of critical thought to planning experiments and interpreting results, and fundamental principles involved in metabolism and growth are not appreciated. The need is great for defining these principles, and synthesizing from reliable data a corpus of integrated knowledge that will enable a better orientation of future experimental work.

An attempt has been made in this review at a natural division of the subject matter into sections concerning absorption and accumulation of ions, the forms in which mineral elements occur in the plant, their significance in metabolism and finally their relation to development and the drift of their content in the plant with age. The division, however, can be only a rough one, since a single paper, necessarily dealt with as a unit, frequently has implications in several of these sections.

ABSORPTION AND ACCUMULATION OF IONS

The accumulation mechanism.—The controversy referred to in a previous review (2) has continued between Lundegårdh on the one hand and Hoagland & Steward on the other (3, 4, 5). Hoagland (6) has also summarized recent investigations on salt accumulation and Lundegårdh (4) in a detailed monograph incorporating new data has set out his general theory of ion intake. To attempt to assess in detail the claims of the two schools would require a critical examination of much early data and would thus be beyond the scope of the present review. Hoagland & Steward show that the results of their own work are incompatible with Lundegårdh's hypothesis; this however, as Robertson (7) has pointed out, may be due to differences in the material used.

Robertson (8), using the conductivity method, has investigated

the accumulation of chlorides by carrot discs and its relation to the respiration rate. The chlorides caused a rise in respiration rate above that of the control tissue in distilled water. With monovalent chlorides the higher rate was maintained, while the rate of accumulation decreased; with divalent chlorides the respiration rate fell again to the control value or below it and electrolyte accumulation was small. In the case of potassium chloride, in spite of differences in accumulation rate in different sets of tissue, the extra carbon dioxide produced per gm. mol. of chloride accumulated tended to be constant for a given amount already taken in. It increased as the latter amount increased, since the rate of accumulation fell with time. The increase in respiration rate was also proportional, although not stoichiometrically so, to the initial external concentration of the chloride; this relation persisted despite fall in external concentration and increase in amount of salt in the tissue. Robertson considers the possible energy relationships concerned, assuming that the entering salt remained in solution and became evenly distributed throughout the tissue. The energy required to accumulate a gm. mol. of salt will increase as the gradient becomes steeper; calculating its actual values from his data for extra carbon dioxide production, Robertson finds that it increased more rapidly in the case of dilute than in that of more concentrated potassium chloride, although the accumulation rate was only slightly less than in the dilute solution. This suggests that the tissue accumulated ions at a rate independent of the concentration of the external solution, so that the tissue appears to have done more work in accumulating from the more dilute solution; the accumulation rate cannot, therefore, be increased appreciably by decreasing the osmotic work necessary for transfer of ions from the external solution. Robertson is able to draw the same conclusions from examination of data of Stiles & Skelding (11) to which we shall refer shortly. He concludes that the accumulation rate must consequently be dependent on some internal attribute of the tissue. He suggests a system wherein one phase of the cell becomes rapidly saturated with the electrolyte, which is subsequently withdrawn into a second phase; the first phase would correspond to an adsorbed film saturated at low concentration on one side. If the initial saturation of the first phase were rapid, the rate of accumulation as a whole would depend on the rate of withdrawal to the second phase, that is, on some factor within the tissue and not on the osmotic gradient between outside and inside solutions. The decrease in rate of accumulation with time would be due to the progressive

saturation of the second phase. Assuming constant concentration in the first phase, the work done in accumulation is found to be linearly related to the extra carbon dioxide produced. Robertson raises the question whether the first phase is the protoplasm and the second the vacuole. With regard to the divalent chlorides it is impossible to say whether their effect in reducing respiration prevents the further accumulation or whether there is another, perhaps toxic, effect. Robertson thus, like Lundegårdh, suggests that accumulation is a function of the energy available from the extra respiration induced by the salt; he does not suggest, however, that it is only anion accumulation that is so related, but points out that cation intake may differ considerably from cation accumulation, since cations much more than anions will enter by exchange.

We must regard Robertson's suggestions as a constructive interpretation of his data, provided we can accept his assumptions concerning the distribution of accumulated ions in the tissue. These, however, are at variance with earlier work of Steward. The latter has shown that intake by discs of storage tissue under his experimental conditions largely concerns cells in the surface layers. These cells become depleted of starch, engage in rapid synthesis of protein and hence of new protoplasm, respire actively, and generally approach the meristematic state; some of them actually divide. The amount of electrolyte taken in thus tends to be merely a measure of the amount of new protoplasm. The final internal concentration in each cell, assuming the new state, might be constant and yet the total intake would vary according to the number of cells so affected, that is, according to the amount of new protoplasm formed. Nongrowing cells can, of course, also take in ions: change in metabolic activity or in external electrolyte concentration may cause intake or exchange of ions as the cells approach a new steady state. It seems that this second type of intake is usually less appreciable in well-aerated discs. But the interpretation of data from discs of storage tissue must remain doubtful so long as the relative significance of these two types of intake is uncertain, that is, so long as it cannot be determined to what extent accumulation occurs per unit of the tissue. Likewise, the critical test of Lundegårdh's hypothesis will depend on selection of material in which ion intake and respiration are not complicated by unmeasured variations in the extensive properties of the system concerned. It is of interest that Hoagland & Broyer (9) have claimed that the growth complication is insignificant in their "low-salt" roots.

Steward & Preston (10) have further used discs of potato tissue in studying the effect of pH and the concentration of a bicarbonate buffer on bromide intake and on metabolism. There was a parallelism between the effects on bromide intake, on respiration, and on protein synthesis. It must be concluded from what has been said above that the treatments affected the growth process, with which respiration and protein synthesis are closely linked. Steward & Preston also observed the second type of accumulation, since they found that tissue in relatively strong bicarbonate solutions achieved neither bromine intake nor protein synthesis, but nevertheless absorbed potassium. They considered that the potassium was absorbed along with the bicarbonate ion, and that the latter reacted with the organic acids of the sap. Their work throws interesting light on various electrolyte effects on the resumption of the meristematic state, and on the close relation between protein synthesis and respiration; it does not, however, appear to advance our understanding of the fundamental mechanism of ion accumulation.

Under conditions that are believed to cause less surface growth, and therefore to permit relatively more intake of the second type, Stiles & Skelding (11) have studied the intake of manganese chloride, sulphate, and nitrate. The manganese intake showed an initial phase of rapid absorption, followed after a period of so-called equilibrium by a prolonged period of slow absorption. Anion intake did not show these two phases: it was at first slower than that of the cations, but later tended to exceed it. The manganese intake differed in several ways from that already reported for potassium [see previous review (12)]: initial intake was more rapid, the end of the first period of absorption was earlier, and the excess of cation absorption was greater. Stiles & Skelding attribute these differences to the fact that the tissue initially contained a considerable amount of potassium, but very little manganese. During the first phase, concentration relationships and apparent conformity with the adsorption isotherm followed on the lines of much previous work on carrot tissue; when, however, the external solutions were renewed, the further absorption of manganese was greater than the requirements of the isotherm. Therefore, the writers conclude that exchange of ions between external solutions and tissue rather than adsorption must play the principal part in determining the amount of manganese absorbed during the first phase. As, of course, has been frequently pointed out, conformity of data with the adsorption isotherm has little theoretical significance, so that

alternative explanations of the results are not excluded. The nature of the second phase of absorption is obscure: Stiles & Skelding suggested in their previous work on potassium that the first phase may represent intake by mature cells, and the second phase that by the surface or growing cells.

Overstreet, Ruben & Broyer (13) consider the question whether roots take in ions by exchange, or whether cations entering the root must be accompanied by an equivalent of anions. They point out that the exchange theory cannot be proved until the roles of hydrogen and bicarbonate ions have been evaluated. Until this is done, for example, it cannot be determined whether, when an apparent excess of cations enters the root, the excess is not accompanied by an equivalent of bicarbonate ions. They have attacked this problem experimentally using potassium bicarbonate solutions containing radioactive carbon. They found that labelled carbon dioxide was absorbed by "low-salt" barley roots and practically the whole of what was absorbed was reduced. This is of interest in view of the recent discovery that the capacity for carbon dioxide reduction in the dark is a general property of cells. The intake of potassium ions, however, was several times greater than that of the radioactive bicarbonate ions. Their work thus supports the exchange theory, but Overstreet and his colleagues point out that potassium might enter the protoplasm accompanied by bicarbonate ions, and that, after the equilibration of the potassium by organic acids, the carbon dioxide might be eliminated. This suggestion, as we have seen, was also made by Steward & Preston. Ulrich (14) has confirmed the fact that, when excised roots absorb cations in excess of anions from solution, organic acids are formed as a response to the tendency towards increase in pH of sap. Conversely when anions are absorbed in excess of cations, the organic acid anions tend to disappear, leaving the base to balance the increase in inorganic anions. On the other hand, Vickery, Pucher, Wakeman & Leavenworth (15) conclude that the intake of ions may itself be considerably affected by variations in organic acid concentration; they found that such variations can be induced by variation in the proportion of ammonium to nitrate ions in the external medium.

Movement of ions in the plant.—Recent papers deal with several aspects of this subject. Mason & Phillis (16) have shown that, when the wood was interrupted, approximately the same proportion of nitrogen absorbed by the cotton plant could move up through the bark as in the normal plants, but the amount absorbed was greatly decreased.

Bromine absorption was also decreased and the plant could not translocate more than a trace through the bark. The writers conclude that so far as nitrogen is concerned this experiment leaves still uncertain the normal path of intake.

Biddulph (17) injected bean leaves with sodium phosphate containing radioactive phosphorus and observed its migration to other parts of the plant. Migration was predominantly downwards. As it proceeded some of the phosphorus apparently passed laterally into the xylem, and was then carried upwards and distributed as if supplied from the external medium; this agreed with the picture of phosphorus movement put forward by Mason & Maskell. Daily periodicity of movement in both directions was observed. The data show that a phosphorus atom, after entering the root system, could easily pass from root to leaf and back towards the root again at least once in twenty-four hours. Various details concerning movement of phosphorus in the tomato plant have also been established by Arnon, Stout & Sipos (18) with the use of the radioactive isotope. After forty minutes newly absorbed phosphorus was detected in the leaves and tips of plants over six feet tall. The pulp of fully ripe fruit continued to take in small amounts of phosphorus, but movement into the seed occurred only when the fruit was green. These authors have also developed a useful technique for producing contact "radiographs" showing the distribution of newly absorbed phosphorus in tissues.

THE FORMS OF MINERAL ELEMENTS IN THE PLANT

Knowledge of the forms or states in which mineral elements occur in the plant and of the variations in the amounts of such forms with age and external factors is a basis upon which the elucidation of the significance of these elements in metabolism must largely rest. For this reason particular interest attaches to a paper by Phillis & Mason (19) on the partition of potassium, calcium, and magnesium in leaves of ten-week-old cotton plants grown with various potassium supplies. Potassium occurred in two fractions which they regard as being sap-soluble and adsorbed; calcium and magnesium had also a third fraction which was chemically combined. As the supply of potassium increased, so also did the partition coefficient (i.e., the ratio of the adsorbed to the sap-soluble fraction). The combined calcium and magnesium were little affected by treatment except in the bottom leaves, where the percentage content of combined magnesium in the dry matter fell with increasing potassium supply; the total calcium

and magnesium, on the contrary, fell greatly with treatment. Thus the poorer the leaves in calcium the greater was the proportion present in the combined form. Noncombined calcium and magnesium behaved similarly to the total. The partition coefficient for magnesium was irregular: for calcium it was positively correlated with the percentage of the noncombined element. The summated base partition coefficient was remarkably constant, calcium and potassium compensating one another. The implication is that the partition of the bases between adsorbed and sap-soluble fractions is determined by base exchange. The results might suggest that the sap-soluble fractions of these elements are concerned in growth and that the adsorbed fractions are luxury consumed, but Phillis & Mason point out that if exchange determines the distribution this seems less probable.

Forms of phosphorus have been studied by Michael & Heidecker (20) in spinach at three growth stages. Inorganic phosphorus and soluble esters occurred abundantly in old leaves with high phosphorus supply, but were low in amount with a deficient phosphorus supply. Alcohol-soluble and nucleoprotein phosphorus varied little with fertilization and were more abundant in the young parts.

Javillier & Goudchaux (21) have determined the proportion of the total magnesium in the plant that is present in the chlorophyll molecule: their results vary from 0.9 per cent in *Pinus maritima* to 26 per cent in *Triticum vulgare*. These proportions must, of course, vary with growth stage and type of nutrition.

In elucidating the metabolic significance of mineral elements not only is their chemical or physical partition important, but also their spatial distribution in the cell. In this connection Hanson, Barrien & Wood (22) have carried out an experiment in which they observed the functions of the total protein nitrogen and sulphur present in the chloroplasts. When the leaves were young, the percentage of the total protein nitrogen present in the chloroplasts was about 7, while it later increased to from 35 to 40. The chloroplast protein was richer in sulphur than that of the cytoplasm, and 70 per cent or more of the protein sulphur was contained in the chloroplasts of the older leaves. The ratio of protein nitrogen to protein sulphur in the chloroplasts was sufficiently constant for the writers to conclude that the chloroplast contains a single protein or a group of allied proteins. After the second harvest the cytoplasmic protein accumulated more rapidly than the chloroplast protein, and this caused decline in the ratio of total protein nitrogen to total protein sulphur with age. As a con-

siderable amount of work is now being performed on isolated chloroplasts it is hoped that the partition of mineral elements between them and the cytoplasm will be further studied.

Reference will be made in the next section to work on soluble and insoluble forms of boron and calcium. Certain properties of the insoluble boron fraction have been noted by Wolf (23).

THE METABOLISM OF MINERAL ELEMENTS AND THEIR RELATION TO METABOLISM IN GENERAL

Work has continued on the effects of varying supplies of mineral elements on amounts of others present in the plant and on other aspects of metabolism. There is a considerable tendency, however, to overlook the principle that direct effects on metabolism may be modified and obscured by the indirect effect that the same treatment has upon certain aspects of ontogeny. As this tendency can lead to misinterpretation of results it has been considered desirable to illustrate the principle by a specific example, taking for this purpose a study by Williams (24) of the relation of growth to phosphorus supply. Increased phosphorus supply to oat plants increased the final dry weight to such an extent that nitrogen supply became suboptimal when that of phosphorus was high. This resulted throughout the later part of the growing period in a decreased protein and total nitrogen content on a dry-weight basis. A deduction from this concerning the effect of phosphorus on protein metabolism would, however, be unsound, since evidence from the early stages of growth and from work of other investigators shows fairly conclusively that increasing phosphorus supply actually increases the rate of protein synthesis. Phosphorus supply probably shifts the position of balance between proteins and amino acids in the direction of increased synthesis; in spite of this, if the supply of nitrogen for amino acid formation declines more rapidly, the percentage content of proteins can eventually be decreased by increasing phosphorus supply. Evidently, therefore, it is difficult to interpret data when they relate to only one point in time and both growth and metabolic effects of the treatment are involved. *Ad hoc* experiments on metabolic effects should involve applying a treatment to initially uniform material and the effects should be followed rapidly over the immediately ensuing period of time; ontogenetic changes may then be slight and effects observed are more likely to be metabolic ones. A technique of this type is discussed by Walkley

& Petrie (25). If conclusions concerning growth effects are to be drawn, they will be sounder if growth is observed progressively in its temporal drift, rather than at a single point in time.

The work discussed in this section also emphasizes how far we have yet to go in unravelling the significance of mineral elements in metabolism, and how various are the roles that they play.

The boron-calcium relationship.—Further work has been carried out on the boron-calcium relationship by Marsh & Shive (26). They found that a large proportion of the boron in maize was in the soluble state, the proportion, as might be expected, increasing with the boron content. Increasing boron supply over the range studied caused the proportion of the calcium in a soluble state to increase from 28 to 54 per cent of the total calcium present. The soluble fractions here correspond in provenance to the "sap-soluble" fractions of Phillis & Mason referred to in the previous section. Marsh & Shive conclude that there is a direct effect of boron on calcium metabolism; in view of the opening comments of this section, however, it is apparent that this conclusion can be accepted only with certain reservations. The amount of growth achieved up to the time of harvest increased greatly with increasing boron supply up to the optimum; in other words the high boron plants were probably at a more advanced stage of ontogeny than the deficient ones. If the proportion of soluble calcium normally increases with development, this might largely explain the results obtained; such an increase is known to occur in the case of nitrogen over certain portions of the growing period, and it might be a reflection of the increase in proportion of sap to cytoplasm with development. A second ontogenetic change that may bear on the data concerns the proportion of plant parts. Marsh & Shive worked with total shoots. The ratio of leaves to stems may have been considerably decreased with boron treatment by the time of harvest. If calcium tended to be fixed in the leaves and to remain free in the stems this could have contributed to the observed relation. This possibility is to some extent dissipated by microscopical observations on the tissues, but these are not quantitatively related to the analytical data. Possible ontogenetic drifts also detract from the significance of relationships that Marsh & Shive conclude to exist between boron content and fats and pectins. These complications in the interpretation of the data may have been unimportant; a different experimental design could nevertheless have obviated the necessity of considering them. Shive (27) obtained similar results with *Vicia faba* except that only

a relatively small fraction of the total boron was soluble. The optimum boron requirements for *Vicia* were considerably greater than for maize.

Drake, Sieling & Scarseth (28) claim that when boron is deficient the plant has a high calcium-boron ratio in its tissues. This does not, however, indicate a close connection between calcium and boron metabolism. So long as the supply of an element is markedly limiting, increased supply may appreciably increase the size of the plant at a given time without greatly increasing the percentage content of the element in the tissues. The content of other elements supplied in constant amount at commencement (in this case calcium) will be greatly decreased as a result of increased size; this has been exemplified above in the case of nitrogen in Williams's work. This principle may explain a number of supposedly significant ratios between mineral elements.

Metabolism of zinc-deficient plants.—Skoog (29) has shown that zinc deficiency caused auxin content to be low in leaves and negligible in terminal buds and stems. Decline in auxin content preceded the appearance of visible deficiency symptoms. From this and other careful observations Skoog concludes that zinc is necessary for a normal auxin content and that the decline was not a secondary effect of decreased vigour. As a further test of this conclusion he compared the effect with those of copper and manganese deficiencies. Lack of manganese produced practically no reduction in auxin content even after severe deficiency symptoms had set in: lack of copper caused a reduction of smaller magnitude than did zinc deficiency, but not until after deficiency symptoms had appeared. Retardation in longitudinal growth in the three types of deficiency was roughly parallel to the decrease in auxin content. Further experiments showed that lack of auxin in zinc-deficient plants must be attributed in part to its more rapid inactivation by the tissues; this is related to the fact that they have a higher oxidation capacity.¹ Under red or weak light, conditions known to be favourable to the stability of auxin, zinc-deficient plants maintained a high auxin content and continued to elongate for long periods. Supplying indoleacetic acid increased stem growth in the early stages of zinc deficiency but it did not replace zinc as a nutrient. Skoog concludes that it increased the capacity for utilizing available zinc but did not substitute the lost auxin. He also con-

¹ Larsen (30) has recently found an oxidizing enzyme in plants that causes the destruction of auxin.

cludes that zinc is not principally required for auxin synthesis but for the maintenance of the normal concentrations in the tissues; this accounts for many of the observed effects of zinc deficiency on plants. The paper is an important one since it may provide a pointer for discovering the actual functional activity of zinc in the metabolism of the normal plant and the way in which this differs from that of copper. It is already known that polyphenol oxidase is a copper protein, and evidence has recently been given (31, 32) that this is the case also for ascorbic acid oxidase. It thus seems that both zinc and copper may be related to the respiratory process but in distinctly different ways.

Function of potassium and its replacement by rubidium.—Richards (33) has made an elaborate and valuable study of the effect of rubidium on the growth of the barley plant, using it as a means to elucidate in part the function of potassium. He has shown that in solutions containing high concentrations of ammonium ions and low concentrations of calcium ions, barley was much more sensitive to potassium deficiency than in other nutrient solutions. With very low potassium levels growth nearly ceased at the first or second leaf stage, and a large proportion of the plants died. Addition of rubidium enabled early growth to proceed almost normally, but if its concentration was too high toxic symptoms appeared. Sodium, lithium and caesium did not have this effect. The effect depended in a complex manner on the calcium, ammonium and phosphorus status of the solution. In the early stages of growth in potassium-deficient solutions, high concentrations of phosphorus tended to be detrimental if the ammonium content was high, but beneficial if the calcium content was high: by the end of the growth period it was always beneficial if rubidium was present, but was usually harmful in its absence. Rubidium was always beneficial when the phosphorus level was high, countering toxic effects shown by careful analysis of the data to be due probably to the high ammonium and high phosphorus levels; but it tended to be detrimental at low phosphorus levels, especially when calcium was high, causing accentuation of the symptoms of phosphorus deficiency. At high phosphorus levels, the percentage content of phosphorus in the plant was higher with high ammonium than with high calcium. With high concentrations of calcium, rubidium always decreased the total phosphorus uptake, but with high concentrations of ammonium ions it increased it, though the percentage content tended to be reduced. In the light of these and other detailed observations, Richards concludes that rubidium may have a threefold effect on the plant. (a) There is a

general toxicity. (b) There is an effect depending on retardation of phosphorus uptake, favourable when phosphorus is excessive and damaging when phosphorus is deficient. In this the behaviour of rubidium is in contrast to that of potassium: rubidium appears to retard the rate of intake of phosphorus; but where increase of potassium lowers the percentage content of phosphorus it is because of the greater growth made by the plant. (c) There is a more doubtful effect, that of countering ammonium toxicity; here the behaviour of rubidium and potassium is similar. Since also in instances where ammonium ion accumulates, the total nitrogen content of the plants was not increased, Richards concludes that the rubidium effect does not result from a general lowering of permeability to ions; on the contrary it appears that rubidium or potassium is essential for the transformation of ammonium ion into innocuous substances as rapidly as it is absorbed. While the evidence points to the probability of this being a real effect, Richards makes it clear that it cannot yet be regarded as fully established. If this effect is real, however, it shows that the total effect of potassium in the plant is analysable into two parts: in one, namely the removal of ammonium, replacement by rubidium is possible; in the other, potassium is unique. Richards (34) has also described a useful method of graphically illustrating the results of experiments of factorial design.

Selenium in plants.—Interest has been aroused recently in certain plants that absorb considerable amounts of selenium from soils containing it in an inorganic state. Horn & Jones (35) have isolated from one of these plants a compound containing both selenium and sulphur and having the properties of an amino acid. They suggest that it may be a combination of two compounds, one containing sulphur and the other selenium. They also suggest that in grain grown on seleniferous soils the selenium is probably combined with protein as an amino acid having a similar structure. This and other evidence indicates a relationship between the metabolism of selenium and that of sulphur; the relationship, however, is not always very close, since Painter & Franke (36) have found that there was neither selenate nor selenite in a plant containing an appreciable amount of sulphate, and also that the ratio of selenium to sulphur was not always constant in grain grown on seleniferous soils.

Relation of mineral nutrients to nitrogen and carbohydrate metabolism.—Beckenbach, Robbins & Shive (37) have continued their work on the relation between nutrient-ion concentration and carbohydrate

and nitrogen contents of leaf and stem tissue of maize. The plants were subjected to differential treatment for forty days, and it must therefore be assumed that growth effects were involved as well as effects on metabolism. This the writers have partly appreciated. They found nitrate ion, ammonia nitrogen, α -amino nitrogen, and protein content increased with nitrate supply and decreased with potassium supply. They attribute these relations to effects on growth rate. Concerning carbohydrate metabolism their conclusions are less acceptable. They found that reducing sugar content decreased with increase in both potassium and nitrate supply. From this fact they make deductions concerning the effect of potassium on energy release in protein synthesis. Examination of results reported by them earlier shows that both increasing potassium and increasing nitrate increased the yield considerably. The variation in reducing sugar content may thus merely have been a reflection of the different growth rate resulting from variation in the limiting level of these two nutrients: where growth is rapid, and there is a rapid deposition of carbohydrates in polymerized forms, it is possible that reducing sugar content is decreased. Beckenbach, Robbins & Shive also assume that the rate of energy release is inversely related to reducing sugar content; this is by no means always true.

Skok (38) has obtained further evidence of an indirect nature that calcium is necessary for the process of nitrate reduction in plants. He found in bean plants that calcium deficiency was less severe in effect when the nitrogen was supplied as urea than when it was supplied as nitrate, although when calcium was present better growth resulted from nitrate.

Eaton (39) has examined the effect of sulphur deficiency on gradients in the stem of the sunflower, mainly to discover whether there is a greater than normal reutilization of protein nitrogen and sulphur. As he eventually concludes, little can be deduced from stem gradients concerning reutilization. By greater reutilization one visualizes earlier commencement of net protein hydrolysis in the lower leaves, and had Eaton observed the drifts in nitrogen content of leaves at different levels he would have obtained results of greater value. Eaton nevertheless tends to regard high contents of soluble nitrogen compounds in the sulphur-deficient stems as implying increased proteolysis. This is unjustified: net synthesis of proteins might be occurring in a tissue, and yet the concentration of soluble nitrogen compounds might be higher, if sulphur deficiency limited the rate of syn-

thesis, than in a nitrogen-limited control; net protein hydrolysis in the leaves may in fact conceivably be delayed by sulphur deficiency. Eaton also concludes that his data suggest a lowering of the reductase content of the plant by sulphur deficiency. Actually there is no evidence for this. If it were so, nitrate would tend to accumulate more than other nitrogen fractions: in reality there was comparatively little more nitrate in the deficient than in the normal plants, although other fractions, namely ammonia, amide, and amino nitrogen did accumulate considerably. It is probably unwise to deduce much from the relations of different nitrogen fractions in the stem, where they may be in different tissues, and in process of translocation during which the system is far from being at a steady state. Nevertheless we are probably justified in drawing the following broad conclusions from the data presented. The sulphur-deficient plants, being inhibited in growth, accumulated nitrogen to a relatively higher degree than normal; this is the same frequently observed principle of mineral nutrition that was exemplified in the work of Williams (24) referred to earlier. If sulphur deficiency inhibits protein synthesis, the data suggest that it may do so mainly by an effect on the final stage, namely that of amino acid condensation. Such an effect could result solely from a lack of sulphur-containing amino acids; thus in the stems of the sulphur-deficient plants the protein content was somewhat less than that of the controls, while the amino nitrogen content was much higher. For a given nitrate content there was also considerably more ammonia nitrogen in the sulphur-deficient than in the normal plants, which is not in agreement with the supposed decrease in reductase activity.

MINERAL NUTRITION AND DEVELOPMENT

Relation to development in general.—The principles of physiological ontogeny in plants are being established only slowly, but it is well recognized that mineral nutrition is one of the most important external factors determining the developmental plan. Watson & Petrie (40) have studied the manner in which phosphorus supply affects developmental change in the tobacco plant. They considered especially dry-weight and leaf-area changes and drifts in the contents of nitrogen and water. The work involved a formulation of ontogenetic principles which is outside the scope of this review; reference may be made, however, to certain effects of phosphorus supply and their interpretation. Increasing supply caused increase in dry weight of roots and

stem; it was suggested that an important factor in this was increased rate of cell division resulting from more rapid synthesis of proteins and essential phosphorus compounds. In the leaves, area was increased more than dry weight; here again greater number of cells may have been a factor, but increase in cell size was suggested as also playing a part. Increased rate of protein synthesis was considered likely to lead to greater expansion of cells, but a direct effect of phosphorus supply on water uptake by the cells was also suggested as a factor. The absolute nitrogen content of all parts was at first increased by increasing phosphorus supply; later the absolute amount in the vegetative parts became less because the proportion of the whole represented by the inflorescence was increased with increasing phosphorus supply. Deficiency delayed migration of nitrogen from the leaves during senescence on account of the smaller demand for nitrogen in plants whose growth was restricted. The percentage of nitrogen in all parts was at first increased but subsequently became less as in Williams's example (24) to which we have already referred. The work of Watson & Petrie is of interest as it shows how the effects of mineral nutrition can be used to reveal the manner of determination of developmental processes.

Loehwing (41) has published a theoretical article on the relation of mineral nutrients to flower production. He rightly stresses the importance of absolute as well as relative data and of "the detailed chronological inventory of the progress of events in root and shoot." In the opinion of the reviewer, however, much of the conception he develops is unsound. The most significant event in the nutritional picture he regards as a sudden increase in the transpiration rate and fall in water content of the tissue at the time of flower inception. Simultaneously the osmotic pressure of the sap increases, and as a result of the reduction in water content there is hydrolysis of insoluble proteins and polysaccharide reserves. In the roots the rate of salt intake decreases. The fall in water content, which, he claims, resembles a trigger action, causes redistribution of salts throughout the plant, depletion of vegetative organs, and concentration of salts in developing stamens and pistils. The fall in water content thus leads to mobilization and translocation to the flower primordia of the elements necessary for their development. No acceptable data, however, have been put forward to show that such sudden lowering of water content occurs at the time of flower inception and there is no evidence at all that such lowering causes the events that Loehwing describes.

Environmental conditions favourable to flower inception may of course frequently be such as independently increase the transpiration rate; but the main decline in absolute water content of tissues is the result and not the cause of senescent processes. There seems little doubt that the primary cause of senescence in leaves, for example, is the rise of extrafoliar sinks such as the inflorescence, which draw on the nitrogen and phosphorus in the leaves and other parts. Much data from the reviewer's laboratory shows that migration of nitrogen from leaves occurs before decrease in absolute water content. The latter decrease is probably mainly caused by protoplasmic breakdown following nitrogen translocation; decrease in water content may eventually accelerate the redistribution of mineral elements within the plant but there is no evidence that it is the initial cause.

Sircar & Sen (42) found that fully manured rice plants with high phosphorus supply produced no inflorescences; on the other hand phosphorus-deficient plants with fewer tillers and reduced vegetative growth produced fertile inflorescences. They suggest that this is due to very high nitrogen uptake by the fully manured plants. Mature leaves were compared in their composition; those of the fully manured plants were high in protein and amino nitrogen, and the contents fell with decreasing phosphorus supply. The leaves of low-phosphorus plants accumulated amides, but were low in nitrogen. This type of sampling gives an inadequate picture of the effect of phosphorus on growth and nitrogen content, as becomes clear when consideration is given to the more exhaustive studies of Williams (24) and Watson & Petrie (40). Sircar & Sen conclude that absorption of nitrogen is dependent on the supply of phosphorus, and that therefore phosphorus is necessary for protein synthesis. This is fairly well established, but it cannot wholly explain their results: they have overlooked the significance of the absence of inflorescences in their fully manured plants. Whatever the immediate cause of this, the absence of such nitrogen sinks would naturally cause a higher percentage content of nitrogen compounds to exist in the leaves, irrespective of any effect of phosphorus on protein synthesis or nitrogen absorption.

Hayward & Long (43) have studied the growth of tomato plants in varying concentrations of a basal nutrient solution and also in a weak basal nutrient solution to which were added varying amounts of either sodium chloride or sodium sulphate. Various morphological effects were observed. In the basal nutrient series, however, effects resulting from variations in nutrient supply must have been super-

imposed on pure concentration effects. Unfortunately also the pH of the culture solutions was sometimes corrected by addition of nitric acid in unstated amounts.

Eltinge (44) has shown that in manganese deficiency the chloroplasts have an abnormally low ascorbic acid content and break down early. Reed (45) has described effects of zinc deficiency on the cells of vegetative buds of fruit trees. The cells were characterized by premature vacuolization, polarization of their contents, and inhibition of multiplication.

Drift of mineral content of the plant with age.—Reference may conveniently be made here to further use by Thomas & Mack (46) of the concept of foliar diagnosis; this is based on percentage drifts of elements in the fifth leaf. It is true that the nutritional status of the plant can be understood completely only by chemical analysis of its parts, and for this purpose analysis of a single leaf undoubtedly has advantages that Thomas and his colleagues have realized. These analyses, however, require to be interpreted in the light of the growth of the plant as a whole; such interpretation calls for consideration not solely of percentage drifts, which are all that Thomas is in the habit of presenting: dry-weight drifts, and drifts of the absolute contents of mineral nutrients are also required. Without these additional data it is often difficult to interpret the data or to accept the interpretations given by Thomas, which too often are marred by jejune conceptions. The main feature of Thomas' method is the presentation on trilinear co-ordinates of the proportion between groups of three mineral elements, generally nitrogen, phosphorus and potassium. These graphs, however, do not appear to possess any value until they are interpreted in fundamental terms deduced from consideration of drifts in absolute and percentage contents and dry weights. When the growth and nutritional status of the plants are so interpreted it is doubtful whether any pragmatic value attaches to this further method of plotting.

Knowles, Watkin & Cowie (47) have presented data showing the drift in composition of the potato plant and its parts with various supplies of nutrient elements, and the redistribution of elements within the plant during growth. In examining such data it is important to bear in mind the principle we have several times reiterated that, if the supply of an element, *A*, is restricted, increasing supply of another element, *B*, tends to reduce the final percentage content of *A* in the plant. If the supply of *A* is not restricted, it is possible that other relationships, including metabolic ones, may be revealed. Knowles

and his collaborators leave much to be desired in the analysis of their data, which reveal effects that cannot be interpreted in terms of this principle. Thus supplying potassium increased the dry weight and decreased the percentage of a number of mineral elements in the plant, but nevertheless increased the percentage content of chlorine. Supplying nitrogen increased the dry weight and also the potassium content, although it decreased the content of other mineral elements estimated; at a higher level of phosphorus this effect on potassium content was absent, although here the dry weight was depressed rather than increased. These results emphasize the fact that many metabolic and antagonistic phenomena must influence the mineral content of the plant in addition to growth effects.

Some light has been thrown on the accumulation of nitrogen and phosphorus by cells in relation to age by Reid (48), and her work may also be of significance in relation to ion intake by roots. She found that in the root apex the absolute amounts of nitrogen and phosphorus per cell increased as long as the cells continued to expand. Blank & Frey-Wyssling (49) have shown that this applies also to protein nitrogen; in other words, during cell expansion the cytoplasm as well as the aqueous phases must increase, a fact that must find an important place in understanding the ion accumulation of the growing cell. During maturation Reid found no further increase in absolute amounts of nitrogen or phosphorus in the cell; on the contrary her data suggest a 5 per cent loss of nitrogen and a 14 per cent loss of phosphorus. The ratio of phosphorus to nitrogen increased during elongation and decreased during maturation. Reid suggests that prior to elongation most of the phosphorus is probably a constituent of nucleoproteins and that during expansion perhaps phospholipids accumulate at the increasing cell surface; it is also likely, however, that inorganic phosphorus is accumulating at this time in the vacuole. Considerable interest would attach to an extension of this promising type of investigation to other mineral elements and also to the case of actively absorbing roots, Reid's roots being grown without nutrient supply.

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PLANT TISSUE CULTURES

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Plants, as well as animals, are characteristically made up of cells aggregated into societies of greater or less degrees of coherence and complexity depending on their phylogenetic position in the evolutionary scale and on their degree of ontogenetic maturity. The metabolism of a plant is, therefore, a summation of the metabolism of a greater or lesser number of these units. In unicellular plants, which are so either because of their phylogenetic position (bacteria, unicellular algae, etc.) or because of their ontogenetic position (spores, gametes, fertilized eggs, unicellular gemmae), this metabolism is relatively simple. In multicellular plants it is a complex integrated sum.

In the mature "higher" plant the metabolic picture is an extremely complex one and conclusions drawn from a study of any given process, carried out on the mature organism as a whole, are subject to unavoidable distortions due to the concomitant influences of a variety of other processes. Sometimes these processes go on simultaneously in all cells and can be segregated only by physiological means. A process with a low optimum temperature can thus be segregated from one having a high optimum temperature by a proper adjustment of the environment. Such a method is seldom if ever fully satisfactory, although it is often the only one available. Sometimes, on the other hand, physiological processes are allocated to specific organs or tissues, as is the case with photosynthesis. Photosynthesis goes on only in tissues containing chlorophyll. The transformation of amino acids into proteins goes on in all tissues. Protein synthesis cannot be adequately studied in an actively photosynthesizing green tissue. The two processes can be segregated by physiological means, but still more satisfactorily by complete anatomical separation of the parts involved. Yet the dissection required to accomplish this, if carried out immediately before study of the processes concerned, may itself result in trauma and other physiological disturbances which must be taken into consideration.

The technique of tissue or organ culture is essentially a method for obtaining an anatomical segregation of parts while permitting the tissues to recover from the traumatic disturbances that usually follow

dissection. Such a dynamic dissection of the organism permits us to arrive at a maximum degree and variety of anatomical and physiological segregation of functions with a minimum of traumatic reaction. It has as its ultimate object the separation and study of each cell as an individual and the controlled aggregation of such individuals into functional groups of progressively increased complexity. This ultimate object has, it is true, not yet been attained with cells of either plants or animals. But much progress has been made and the technique is already a useful tool for the study of the physiological processes going on in living materials. As applied to plant tissues, the technique has been employed to date only on the Phanerogams and, with a few exceptions, mainly on the Dicotyledons.

The history of plant tissue cultures has been adequately reviewed elsewhere (1 to 7) and need be recapitulated here only very briefly. The concept was first clearly formulated in 1902 by Haberlandt (8). Cultures of excised plant tissues or organs (roots) capable of brief but not unlimited survival and growth were reported in 1922 by Kotte (9, 10) and by Robbins (11, 12). The first cultures shown to be capable of theoretically unlimited growth were reported in 1934 by White (13), using roots of tomato. The first true tissue cultures were established in 1939 by White (14), by Gautheret (15, 16), and by Nobécourt (17). Since 1935 the technique has developed rapidly and has been applied to the study of a variety of problems, many of which are of interest to biochemists.

The biochemical aspects of tissue culture studies may for convenience be divided into a number of categories. Some obvious ones are: inorganic nutrition; organic nutrition—carbohydrate; organic nutrition—nitrogenous materials; vitamin requirements; hormone relations; enzyme relations and respiration; pathological agents; neoplasia; and morphogenesis. These topics will be taken up in sequence.

Inorganic nutrition.—One of the first and most important problems underlying the development of a successful tissue-culture technique was obviously a study of the inorganic requirements of excised tissues. The requirements of intact plants are, and have been for many years, fairly well known and formed a foundation on which to build, but it was by no means certain that the requirements of a given tissue would be the same as that of another tissue or of the organism as a whole. Bones obviously require somewhat different inorganic nutrition from heart muscle or pancreas. Haberlandt (8) used in his preliminary experiments a modified Knop solution, to which were added glucose

or sucrose, glycerin, asparagin, and sometimes peptone or tissue digests. Robbins (11, 12) employed a Pfeffer's solution, with dextrose or levulose, peptone, asparagin, creatinin, glycocoll, or autolysed yeast added. White (18, 19, 20) began with the inorganic formula of Uspenski & Uspenskaia (21), because it permitted a wider range of pH changes without precipitation than did Pfeffer's, Knop's, or other solutions (20). After a careful study (20), this was modified by replacing the potassium carbonate with potassium chloride, resulting in a solution resembling a dilute Pfeffer's solution with greatly reduced phosphate content, and with dextrose and yeast extract added. This solution proved satisfactory for preliminary studies on roots of wheat (18, 19, 20), seed primordia of snapdragon (18), embryos of purslane (18), and stem tips of chickweed (22), and, as regards the inorganic constituents, was satisfactory for roots of tomato (13) and for a variety of other dicotyledonous plants (23). A comparison with a number of other inorganic nutrient solutions (24) showed it, for this purpose, to be equal to or superior to all others tested. When, however, the yeast extract was replaced by synthetic materials of known composition and lesser complexity (25, 26, 27, 28), with the consequent elimination of unknown "trace" elements contained in the yeast, the solution proved to be no longer satisfactory and had to be supplemented with certain accessory salts (28 to 32). Bonner & Addicott (33) developed a slightly different inorganic solution for use with pea roots. Robbins & Schmidt likewise studied the question in some detail (34, 35, 36), paying particular attention to the possibility of substituting ammonium salts for the nitrates [see also (20)] and the essential character of the sulfate radical. All solutions now in use contain a considerable number of elements. Plant tissues, although they require most of these, seem to be relatively indifferent to the exact concentrations of some, namely: oxygen, hydrogen, nitrogen, calcium, potassium, magnesium, and sulfur. Iron and phosphorus are also required but are tolerated only at very low concentrations. Iron is supplied either as the sulfate (20), chloride (34), citrate, or tartrate (30, 33). White (20) found the sulfate to be superior to other inorganic forms. Robbins & Schmidt (34) came to the same conclusion, but attributed the result to impurities in the sulfate. White added to the above list manganese, boron, zinc, iodine (28), and sodium (unpublished). Bonner & Addicott (33), Robbins & Schmidt (34), Robbins *et al.* (29), Gautheret (15, 16), and Nobécourt (17, 31) concurred in the use of manganese, boron, and zinc. Gautheret

and Nobécourt likewise added copper, cobalt, nickel, titanium, and beryllium, basing this on the usage recommended by Berthelot (32). There seems to be no experimental evidence to support this usage. Robbins *et al.* (29) studied the effects of copper and thallium and rejected these elements as without significance. White, in earlier work (24, 25, 26, 27), used the accessory salt solution of Trelease & Trelease (37) containing in addition to elements listed above, silicon, aluminum, and lithium, but later work (28) showed these to be unimportant or injurious. The inorganic nutrient used at present with complete satisfaction in this laboratory for the cultivation of excised tissues from tomato, tobacco, and sunflower has the following constitution:

MgSO ₄	360 mg.	MnSO ₄	4.5 mg.
Ca(NO ₃) ₂	200 mg.	Fe ₂ (SO ₄) ₃	2.5 mg.
Na ₂ SO ₄	200 mg.	ZnSO ₄	1.5 mg.
KNO ₃	80 mg.	H ₃ BO ₃	1.5 mg.
KCl	65 mg.	KI	0.75 mg.
NaH ₂ PO ₄	16.5 mg.	H ₂ O	1000 ml.

Conclusive evidence is available that all elements in this solution, with the exceptions of sodium, chlorine, and iodine, are essential. Sodium has been added in order to increase the sulfate concentration without increasing the concentration of calcium. Chlorine has been added for the purpose of increasing the potassium concentration without increasing the concentration of nitrate. The addition of iodine is based on as yet not fully conclusive evidence of its desirability (28).

Organic nutrition—carbohydrate.—Plant tissues require for their normal maintenance a source of energy in the form of carbohydrate capable of being respired. In the intact plant this is supplied by the photosynthetic tissues, in the form of dextrose, sucrose, or some other sugar or sugars. In cultures of non-chlorophyll-bearing tissues this requirement must be met from the nutrient solution. The second requisite for obtaining successful plant tissue cultures, after setting up a satisfactory inorganic nutrient, was therefore the development of a satisfactory substitute for the natural supplies of sugars. Haberlandt had used sucrose (8), Kotte, dextrose (9, 10). Robbins compared the effects of a number of sugars on the growth of corn roots and concluded that the most satisfactory ones were, first, dextrose and, second, levulose (11). White likewise used dextrose in early studies on wheat (18, 19, 20), *Stellaria* (22), *Antirrhinum* (18), and *Portulaca* (18). In studies on roots of tomato, dextrose proved un-

satisfactory (13), and a survey of a series of sugars (arabinose, mannose, galactose, glucose, fructose, sucrose, lactose, maltose, raffinose, dextrin, and inositol) showed sucrose to be outstandingly superior for this plant (13, 38, 39). Bonner (40), Bonner & Addicott (33), Malyshchev (41), Fiedler (30), and Thielman (42) likewise found sucrose superior to other sugars for several species of plants. Robbins & Bartley (43) concluded that sucrose and dextrose were of about equal value for tomato roots of two distinct strains. Robbins & Schmidt later recommended maltose and brown sugar as sources of carbohydrate (34, 35, 36) for tomato roots. These appear, however, to owe their beneficial effects largely if not entirely to their content of impurities such as pyridoxin (see later). Mannose has been tested by White (38), Robbins & Schmidt (34), and Gautheret (44, 45). Robbins & Schmidt found it beneficial for their strain of tomato roots, while White found it injurious to another strain of tomato roots and Gautheret found it toxic to cambium of elm. Robbins & Schmidt also examined cellobiose (34), and Robbins *et al.* (29) studied xylose without finding any beneficial effects. Since dextrose is not superior to sucrose for any tissues yet studied in detail and is very difficult to obtain in pure form (36, 42), while sucrose is easily purified, dextrose has been for the most part discarded. At present sucrose is employed as source of carbohydrate in almost all plant tissue culture work in preference to all other sugars. The proved availability of sucrose as an energy source for a variety of excised plant members raises some interesting questions on permeability and on transport and digestion which have not yet been satisfactorily answered.

Organic nutrition—nitrogenous materials.—As indicated above, excised plant tissues are able to satisfy the major part of their nitrogen requirements at the expense of the inorganic nitrates of calcium and potassium contained in the basic nutrient. Robbins & Schmidt (34, 35, 36) believe that no other nitrogenous materials are necessary. Certainly excised roots of tomato can be grown for long periods in a nutrient containing only salts, sucrose, and thiamin (see later) (46 to 50). But growth under these conditions is at a low level and obviously abnormal. Nitrogenous materials of unknown significance were present in all the early nutrients used for plant tissue cultures in the form of peptone (8, 9, 10, 12, 51), meat extract (9, 10), fibrin digest (18), yeast extract (12, 13, 20, 30, 33, 40), or some form of tissue extract (9, 10, 51, 52, 53). Analysis of the yeast extract indicated the importance of the amino acids (25, 26, 54), and both White

(26) and Bonner & Addicott (33) developed amino-acid mixtures to supply this need. White later found that glycine (55) would satisfy the entire requirements for roots of tomato and sunflower but not for roots of clover. Robbins & Schmidt (34, 36, 50), Bonner (40), and Bonner & Devirian (56) were unable to verify the utilization of glycine under their conditions of experiment. Robbins & Schmidt recommend the use of pyridoxin (35, 36, 50), a nitrogen-containing vitamin, in place of glycine. Bonner (40) and Bonner & Devirian (56) support this usage and add nicotinic acid, another nitrogen-containing vitamin, as being required by roots of tomato, pea, and radish, but not by flax. Glutathione (53), nucleic acid, pimelic acid, pyruvic acid, asparagin (34, 36), adenine (56), and folliculin (57) have all been tested, with negative results. Gautheret found some beneficial effects from adding cysteine to nutrients for roots of pea and cambium of willow (4, 58). The exact requirements of plant tissues as regards organic nitrogen are still obviously in doubt. Analyses have not been made to show how much nitrogen disappears from amino acid, pyridoxin, or nicotinic acid solutions on cultivating plant tissues therein. It is clear that plant tissues do benefit from the presence of some form of organic nitrogen in the nutrient but that they can survive for long periods and make some growth without them. Beyond that, little can be considered definitely established.

Vitamin requirements.—The yeast extract used in much of the early work on plant tissue culture (11, 13, 20, 22, 30) provided unknown materials and must have included traces of all of the vitamins of the B complex, as well as others. Bonner (46), Robbins & Bartley (48), and White (27) concluded almost simultaneously that thiamin was one of the substances of prime importance. The significance of thiamin has since been thoroughly established. Robbins & Bartley (49) early concluded that only the thiazole portion of the thiamin molecule had to be supplied in the nutrient for tomato roots, the tissues synthesizing their own pyrimidine. Bonner (59), and Bonner & Buchman (60), on the other hand, found that pea roots required both the thiazole and the pyrimidine, in equimolecular quantities. Some plant tissues, notably those of carrot roots (Nobécourt, 61), can synthesize thiamin from the inorganic elements. It is now generally conceded that all plant tissues must have an adequate supply of thiamin for normal growth. Whether or not thiamin must be supplied from without, and what degree of complexity of its constituents is required before these can be utilized by the tissue is a matter which must

apparently be decided specially for each tissue. Its function is still a matter of conjecture (62).

While thiamin is the only vitamin universally recognized as being essential for the normal functioning of plant tissues, there are others that have been suggested as essential for or at least beneficial to some. Robbins & Schmidt have presented evidence of the importance of pyridoxin (vitamin B₆) for roots of tomato (35, 36, 50), and Robbins (63) has shown a genetic difference in the capacity of different strains of tomato roots to utilize pyridoxin. Bonner has verified the conclusion that pyridoxin is beneficial to some strains of tomato roots and concluded (40), in contrast to White (55), that glycine (see above) was without significance for his strains of tomato roots. White (64) found no evidence of any effect from pyridoxin under his standard conditions. Bonner (40), Addicott & Bonner (65), and Addicott & Devirian (66) found nicotinic acid to be important for roots of tomato, pea, and radish, replacing the amino acids in the nutrient, and Bonner (67) found that it stimulated growth of pea embryos. Robbins & Schmidt, on the other hand, could detect no beneficial effects of nicotinic acid or nicotinamide on tomato roots (36). Vitamin B₂ (lactoflavin) (36, 56), vitamin C (ascorbic acid) (36, 57, 68), vitamin E (56), vitamin G (36), vitamin K (56), urea (36), blastanin (30), inositol (25, 33, 36), pantothenic acid (36), and β -alanine (69) have all been tested on one or more tissues and shown to have little if any beneficial effect. Biotin has been shown to be beneficial to the growth of excised pea embryos (57, 70). Unidentified substances beneficial to the growth of roots of tomato were found in filter paper by Robbins & V. B. White (53) and by Robbins *et al.* (29).

It is to be concluded that thiamin or its precursors and in addition some other material such as glycine, pyridoxin, nicotinic acid, biotin, etc., are necessary for growth of most plant tissues and are usually not synthesized by such tissues. Other vitamins may be required, but if so are generally supplied through the synthetic activities of the tissues themselves.

Hormone relations.—The literature of "plant hormones" has grown enormously in recent years. The cultivation of excised plant tissues and organs, and especially of roots, *in vitro*, offered a new method of studying the essential nature of and behavior towards plant growth substances. Fiedler (30) concluded that, while auxin was present in root tips at the time they were excised, it disappeared very rapidly, by basipetal movement and oxidation at the cut surface, and

that the roots continued to grow normally and to react geotropically in the complete absence of auxin. Boysen-Jensen (71) questioned the reliability of Fiedler's methods of extracting and testing for auxin. Fiedler also found that indoleacetic acid, added to the nutrient, depressed growth at all save extremely low concentrations but gave a possibly significant stimulation at proper and low concentrations. Geiger-Huber (72) and Geiger-Huber & Burlet (73) concluded that heteroauxin definitely stimulated root growth of maize, while Leonian & Lilly (74), Bouillenne (75), and Thielman & Pelece (76) found that it inhibited growth, bringing about marked distortion and abnormal developmental patterns. Fiedler (30) found the related rhizopin and rhizocalin to inhibit growth of excised roots. Nagao, on the other hand, found that roots not only were benefited by addition of traces of indoleacetic acid to the nutrient but also secreted auxin while growing in culture (77, 78, 79). Duhamet found heteroauxin to stimulate growth of roots of lupin (80). Van Overbeek (81, 82) and van Overbeek & Bonner (83) undertook to re-examine the subject and concluded that, while the concentration of auxin in roots did show a marked decrease after excision as had been reported by Fiedler (30), auxin did not disappear entirely, the concentration falling off to a constant low level at which it remained through repeated passages. They concluded that excised roots synthesize their own auxin in culture but in amounts much smaller than those normally supplied to these organs by other parts of the plant.

Nobécourt (31, 84) and Gautheret (15, 16, 85, 86, 87, 88) concluded that an external source of indoleacetic acid was necessary for normal growth of carrot tissue. La Rue found it beneficial to the growth of tissues excised from embryos of various plants (89, 90). White, on the other hand, has grown tissue cultures of *Nicotiana* (91, 92) and of sunflower (93) for long periods without any external source of auxin, and Thimann & Skoog (94) showed that auxin was formed in *Nicotiana* tissue cultures. The true position of auxin in the economy of excised plant tissues must still be considered uncertain.

Haberlandt long ago adduced evidence of the existence of a wound hormone in plants (95, 96). Bonner & English have recently studied this question, have extracted from bean tissues a substance which they call "traumatin" which they identify with Haberlandt's "Wundhormone," and have tested its effects by a modified tissue culture method (97, 98, 99). Duhamet studied the effects of colchicine on growth and mitoses in excised roots of lupin (100). Loo & Loo

found in plant tissues unidentified material which stimulated growth and which they considered to be a hormone (101, 102). An interesting point brought out by their work was the injurious effect of extracts of plant tissues containing tannins. They give no explanation of this effect, but it seems likely that the tannins precipitated and rendered unavailable the iron of the nutrient.

Enzyme relations and respiration.—Essenbeck & Suessenguth (103) early used a modified tissue culture technique in the study of enzyme secretion by germinating corn embryos. Robbins & Maneval (104), Fiedler (30), White (19, 92), and Gautheret (4, 44, 45) have studied the effects of oxygen supply on growth and differentiation of various plant tissues. The respiration of tissue cultures of *Salix* cambium was studied in a preliminary way by Plantefol (105) and that of carrot tissues by Plantefol & Gautheret (106). Respiration seems to be involved in the processes of water secretion (107 to 111) and of salt absorption (112, 113) by excised roots and of differentiation in tissue cultures (44, 45, 52, 92, 114). It is obviously involved in the general processes of growth. Its study by means of tissue culture techniques bids fair to prove of very great importance. While potentially this technique is valuable for such a purpose, most workers in the field of plant tissue cultures have, to date, been too preoccupied with nutritional problems to develop the broader physiological aspects except incidentally. Plant roots, and tissue cultures, both of roots and other materials, having small diameters and apparently normal behavior, offer certain marked advantages over most other plant materials available for the study of respirational and enzymatic processes.

Pathological agents.—The formation of bacterial nodules on the roots of legumes was studied some years ago by means of a simple organ culture technique by Lewis & McCoy (115) but has not been approached by the more recent and more highly developed techniques. The technique of root cultures was early employed by White as a means of maintaining plant disease viruses *in vitro* (117). The root culture method has the advantage of separating the process of virus multiplication from the color pattern manifestations by means of which the presence of viruses is commonly diagnosed. The behavior of the virus is thus not complicated by other processes. The relations between virus multiplication and host tissue multiplication, rate of virus movement, and tissue symptoms were studied in this manner (116, 117). Advantage of the method was taken by Stanley (118) to demon-

strate that aucuba mosaic virus which had multiplied for long periods in chlorophyll-free tissues was chemically and physically indistinguishable from that produced in the presence of the chlorophyll apparatus.

Neoplasia.—Erwin F. Smith early recognized the potential value of a tissue culture technique in the study of plant neoplasia and did all in his power to encourage the development of such a technique. Unfortunately, he did not live to see its consummation. Riker (119), Riker & Berge (120), Berthelot & Amoureux (121), Nobécourt (122), and Nobécourt & Dusseau (123) likewise envisaged such an application. Recently, White & Braun (93), taking advantage of the native sterility of the secondary crown-gall tumors developed on sunflower (124), have succeeded in isolating and cultivating *in vitro* tissues of varying degrees of malignancy, some of which are capable of producing bacteria-free crown-gall tumors when grafted back into sunflower or artichoke after prolonged multiplication *in vitro*. The tumor-forming capacity has thus been segregated from the bacteria and the tumor can be studied and its metabolic processes followed in the absence of the original inciting agent. These experiments, at present only in their beginning, would seem to open up great possibilities for the study of the mechanisms of neoplasia.

Morphogenesis.—A desire to study the processes of morphogenesis was the basis for most of the attempts to develop a tissue culture technique (1, 2, 3, 8). The method has already found a number of important applications in this direction. Tukey, in his studies on the development of excised embryos from "physiologically sterile" varieties of hybrid peaches and cherries (125, 126), has contributed important information on the shifting carbohydrate requirements at different stages of development. La Rue (90) and La Rue & Avery (127) have followed the development of excised embryos of *Zizania*, largely from the morphological point of view. Galligar has studied the growth of roots taken from seeds having different types of storage foods—starchy, fatty, or proteinaceous (128, 129). The most careful studies of morphogenesis using true tissue cultures are those of White (92), Gautheret (44, 45, 52), and Nobécourt (114). White showed that differentiation of callus cultures of *Nicotiana* was promoted by conditions which were correlated with, if not dependent on, a reduced oxygen tension (92). Gautheret examined the factors involved in the differentiation of buds on cultures of cambium of *Ulmus campestris* (44, 45), while Nobécourt studied the rooting of cultures of carrot callus (114).

The review presented above of the present status of the plant tissue culture field from the biochemical point of view, cursory as it necessarily is, reveals a rather chaotic state. The facts that have been clearly and unequivocally established are meager by comparison with those which have been merely suggested. Nevertheless, the extremely wide range and variety of problems which have been or can be attacked by means of such a technique is evident. Certain laboratories, especially those of Gautheret at Paris, Nobécourt at Grenoble, White at Princeton, Robbins at New York, and Bonner at Pasadena, have made extensive and effective use of the method in the solution of biochemical problems. It is not essentially a difficult technique, and it is to be hoped that other laboratories will find occasion to make use of it in the future.

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IMMUNOCHEMISTRY

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During the years immediately succeeding the last world war the interest of immunochemistry was focused mainly on the chemistry of antigens and the relation of specificity to chemical structure. Now, however, that a large volume of evidence has accumulated which shows that antibodies are modified serum globulins, the chief problem has become the structure and properties of proteins in general and the special characteristics of antibodies in particular. Modern methods of investigation are beginning to yield valuable information about both these questions.

Physical characteristics of antibodies.—The distribution of antibodies among the fractions of serum globulin that can be separated by electrophoresis was noted briefly in the last review (1). It was then mentioned that, in horse antipneumococcic serum (Type I) antibody appears to be in a special globulin fraction (T-globulin), migrating between β - and γ -globulin. Since then several investigations have been reported (2 to 9). Van der Scheer, Wyckoff & Clarke (3), at the Lederle Laboratories, recognize three groups of sera, characterized by (a) enhancement of γ -globulin and no measurable amount of T-globulin, (b) considerable T and approximately normal γ , and (c) increased γ , together with more or less T.

In general all the antitoxins examined contained T, while the essentially anticarbohydrate and antiprotein sera, such as antipneumococcic, antimeningococcic, and antihæmorrhagic septicaemia sera, had high γ -fractions. This conclusion conflicts with that of Tiselius (4) and of Tiselius & Kabat (5). In the latest report from the Lederle Laboratories (6), on sera of horses immunized against pneumococci of Types I, II, V, VII, VIII, and XXIII, a T fraction was found in only one instance. In the other antipneumococcic sera some 66 to 74 per cent of the γ -globulin fraction was antibody. Pappenheimer (7), Kekwick (8), Van der Scheer (3, 3a) and their colleagues agree in placing a considerable proportion of antitoxic globulin in a new fraction (T or β_2), with a mobility between that of the γ - and the β -fractions. Van der Scheer and colleagues (3a) found a close relation between the appearance of a large T component and high antitoxic

activity in antitetanus serum. According to Kekwick (8, 9) the proportion of antitoxin contained in the γ -fraction is higher in anti-Welchii than in antidiphtheria sera, and in antitetanus sera rises to 50 per cent. As immunization proceeds the proportion of diphtheria antitoxin in the new (β_2) fraction rises. Electrophoretically pure β_2 -fraction differs remarkably from the γ -fraction; it flocculates more slowly when mixed with toxin and has a lower $L+ / L_t$ ratio. This agrees with the lower flocculation rate (at constant toxin concentration) of sera from horses after prolonged immunization and with the observation of Barr & Glenny (10) that the least soluble fractions of serum (containing the higher proportion of γ -globulin) have a higher $L+ / L_t$ ratio. Also the floccules formed in the most rapidly flocculating mixture of toxin and γ -globulin contained 0.0032 mg. of antibody N per L_t , while those formed with β_2 -globulin contained 0.0016 mg. [the value found by Pappenheimer & Robinson (11) and by Pope & Healey (12)].

Van der Scheer, Wyckoff & Clarke (13) found that when diphtheria and tetanus antitoxin were subjected to digestion, which did not reduce the ability to neutralize toxin, the T component was converted into a slower component, that moved at substantially the same rate as γ -globulin. The abnormal charges, that confer on this T fraction of antitoxin its special mobility in an electric field, are not therefore necessary for its specific activity.

The globulin fractions, obtained by precipitation with ammonium sulphate, do not contain one electrophoretically pure species, although γ -globulin appears mainly in the less soluble fractions. The water-insoluble fraction contains both β - and γ -globulin. As antibodies are confined to the γ -fraction, T-fraction or both, it is not to be expected that pure antibody would be obtained by fractionation with salts or by separation of water-insoluble globulin. The purified diphtheria antitoxin used by Pappenheimer and colleagues (7) in their studies of molecular weight and mobility was essentially the fraction of water-soluble globulin precipitated by half saturation with ammonium sulphate and not precipitated by one-third saturation. It contained some 25 per cent of α -globulin and 75 per cent of the new component. Over 30 per cent of the total protein was precipitable by toxin.

Raffel, Pait & Terry (14) studied the distribution of antibodies among the water-insoluble fractions by periodically removing the precipitate formed during prolonged dialysis.

The size and shape of antibody molecules.—From further studies

(2a, 6, 7, 15) with the ultracentrifuge and of rates of diffusion and of viscosity, it has been concluded that antibodies can be classed into two groups, (A) those with molecular weights about the same as those of normal serum globulins (about 150,000) and (B) those with molecular weights about 900,000. Group A includes all antibodies in rabbit, monkey, and human sera that have, so far, been studied; while only antipneumococcic antibodies in the sera of horses, cows, and pigs are included in Group B. Diphtheria antitoxin in horse serum falls into Group A (7).

Kabat (15) found that smaller antibody molecules appeared in the serum of horses after prolonged immunization with pneumococci. However, Van der Scheer, Lagsdin & Wyckoff (6) saw no evidence of smaller molecules in thirteen antisera against six types of pneumococci. All the heavy fraction was precipitable with polysaccharide; still heavier components that appeared in these sera were not removed by the homologous polysaccharides.

These estimates of molecular weights have allowed the establishment of the actual molecular proportions in antigen-antibody precipitates. They have also made it possible to calculate the amounts of antigen or antibody bound in zones in which no precipitate is formed (7). Estimates of the dimensions of the molecules are needed in considerations of the influence of spatial relations on the formation of antigen-antibody complexes. Although serum proteins are described as "globular," in contrast to the fibrous proteins, it is probable that they are far from spherical. Attempts have been made to calculate the ratio of length of protein molecules to their diameter (a/b), usually with the assumption that they are ellipsoids of rotation (16). The validity of the assumption on which these calculations are based is doubtful. The degree of hydration of the molecules is not exactly known. Neurath, Cooper & Erickson (17) calculate that the ratio a/b of the supposed prolate ellipsoid of serum globulin is 7.2 with no hydration and 5.2 with 33 gm. of water of hydration to 100 gm. of protein. Crowfoot (18) considers that, in crystals, the molecules of insulin and lactoglobulin may be nearly spherical, while haemoglobin is a triaxial ellipsoid with a long axis 1.63 times as long as the short axis. While Neurath and colleagues (17) calculate from viscosity data that the ratio a/b of lactoglobulin is 3.2 even with 33 per cent hydration. Also, according to Crowfoot, the unit cell of crystals of insulin cannot accommodate a molecule with the shape of a prolate ellipsoid.

However, the decrease of calculated asymmetry with increasing hydration becomes less when the molecules are highly asymmetric. As horse antipneumococcal antibodies show streaming birefringence (15) they must be rod-shaped, and it is probable that the length is some twenty times the breadth. Accepting 33 per cent hydration and a value of 5.2 for the ratio a/b , the length and diameter of an antibody molecule, of molecular weight about 150,000, are about 250 Å and 45 Å respectively.

Measurements of the thickness of films on solid surfaces (19 to 24) offer a new approach to the study of the specific union of antibodies and antigens and should give the size of the reacting molecules in one dimension. The type of film used may be either one picked up from a water-air interface onto a solid surface (thin layers) or a film spread from a solution onto a solid surface (thick layers). In films picked up from a water-air interface protein molecules are spread in layers one amino acid thick, whereas in films spread on solids, from solution, the molecules retain a more or less "globular" form. On the other hand polysaccharides form very thin layers when spread from solution.

Both with pneumococcal polysaccharides (21) and with catalase (22) as antigens, alternate layers of antigen and antibody can be deposited (up to five layers of antibody in the catalase-anticatalase reaction), showing that antigen and antibody are polyvalent with respect to each other.

The relatively thick films of protein antigens, obtained by the second method, bind antibodies. This adsorption is not entirely specific; films of diphtheria toxin adsorb not only antitoxin but also egg albumin and nonspecific proteins of horse serum (21). It appears that more of the antibody is adsorbed. Type specific polysaccharides are adsorbed specifically on thick layers of homologous antibody, and these layers of antigen adsorb specifically a further layer of the homologous antibody (21). Thick films of diphtheria antitoxin will not bind toxin; Porter & Pappenheimer (21) ascribe this to an asymmetric distribution of the binding sites on antitoxin molecules.

When pneumococcal polysaccharides are adsorbed on layers of antibody they form thin spread-out films; these thin films will bind antibody. This is not surprising as the determinant groups of the polysaccharide (cellobiuronic acid of S III, for example) will not be deformed by spreading. However, according to Rothan & Land-

steiner (23) and Bateman and colleagues (24), thin films of egg albumin will bind the corresponding antibody; and Harkins and colleagues (22) find that the protein catalase, when adsorbed on layers of antibody, forms a film only about 10 Å thick and that these films will still bind antibody. Bateman and colleagues (24) also found that layers of M substance of streptococci, spread by the first method, bound antibody specifically. However, antigen molecules in these layers were not spread out completely; the minimum thickness obtained was 14 Å. The determinant groups of these proteins, therefore, survive gross deformation of the molecules. This would not be expected from the effect of various methods of denaturation on specificity. On the other hand, Danielli, Danielli & Marrack (25) found evidence that thin films of antibodies would not react with antigens.

Although all have been of the same order, the dimensions of antibodies deduced by various workers from the thickness of films have varied considerably. Porter & Pappenheimer (21) attribute these discrepancies, in part at least, to the methods of measurement employed. The calculated thickness will also depend on the completeness of the film; values will be low if some parts are incompletely covered. Bateman and colleagues (24) calculate the fraction of the antigen film covered by antibody. They assume that after repeated application of antiserum the surface is completely covered. Calculated in this way, the time required for half the active surface to be covered by antibody varies from five minutes with undiluted serum to thirty minutes with serum diluted 1 to 300. When undiluted serum was applied the final thickness of the antibody layer was about 150 Å, while if the serum was diluted 1 to 300 before application the deposited antibody was only about 50 Å thick. The higher figure is less than the calculated long axis of a pseudoglobulin molecule, assumed to have the form of a prolate ellipsoid, with allowance for hydration. If concentrated serum was applied after dilute, there was no increase of thickness. Bateman and colleagues consider that variation in thickness of the antibody layer is due to differences in the orientation of antibody molecules. It may be supposed that, when small amounts of antibody are applied, the molecules are attached by more than one binding site to the antigen layer; while antibody, if in excess, is attached at only one point.

The deposition of antibody on films of M substance in which the water-soluble groups were towards the plate were compared with deposition on films in which the water-soluble groups were free. No

appreciable difference was found. It was considered probable that some rearrangement of groups may occur while the films are in contact with antibody.

Anderson & Stanley (26a) have been able to follow, with the electron microscope, the changes produced in the appearance of tobacco mosaic virus by treatment with rabbit antiserum. One hour after treatment the rod-shaped particles appeared denser and their thickness increased from about 15 to about 60 m μ . The authors suggest that the antigen particles are covered by a layer of elongated antibody molecules attached by their ends. However, if this increase of thickness were due to antibody (even if the covering layer were complete) the ratio of antibody to antigen in the precipitate would be much higher than that actually found by Kleczkowski (31) at extreme antibody excess. It seems possible that these thickened rods consist of side to side aggregates of particles (26b), fixed by links of antibody. The larger aggregates formed later are irregularly arranged and are such as might be formed by nonspecific flocculation.

The formation of antigen-antibody complexes.—The studies of antigen-antibody reactions by means of the ultracentrifuge and in films have proved that both antibody and antigen are polyvalent with respect to each other; and the specific formation of alternate layers of antigen and antibody shows that a complex can be formed containing many molecules. How far a similar specific combination in three dimensions accounts for the formation of precipitates is still undecided. It is possible that the size of such complexes is limited and that further aggregation is due to nonspecific flocculation of hydrophobic particles. Miles & Pirie (102) found that under certain conditions the complex of the antigen of *Br. Melitensis* with its antibody formed plates with a definite mutual orientation; further aggregation was irregular.

Harkins and colleagues (22) found that the enzymic activity of a layer of catalase was little affected by a superjacent layer of antibody. Molecules of hydrogen peroxide must therefore have free access to the catalase through the layer of antibody molecules. These cannot be tightly packed even when the antigen molecules are presented in the more or less orderly arrangement of a surface layer. It would probably be better, as suggested by Pauling (27), to discourage the use of the word "lattice" to describe these aggregates of antigen and antibody, because of the regularity associated with it. It has been considered that the antigen-antibody precipitate became less soluble

on keeping; this might be attributed to a progressive increase in the regularity of arrangement of the molecules in the complex. However, Boyd (28) found that the precipitates of egg albumen and anti-egg-albumen, when stored in the ice box for ten months, were as soluble in excess of antigen as were fresh precipitates.

Pauling (27) has discussed the formation and specific reactions of antibodies from a theoretical standpoint. He assumes that the polypeptide chains of antibody molecules do not differ from those of normal serum globulin either in the amino acids that they contain or in the order of these amino acids in the chains; that the difference between antibody and normal globulin lies in the way in which these chains are coiled in the molecules. He suggests that, in the course of formation of an antibody molecule, the middle part of the polypeptide chain is fixed, while the ends are free; that an antigen molecule may become fixed in such a position that these two free ends adapt themselves to certain configurations on the antigen molecule; and that when the antibody molecule becomes free the two parts of the molecule formed from these two ends remain so coiled as to be adapted to combination with antigen. Pauling also considers the ratio of antibody to antigen in the precipitates formed in the zones of equivalence and at extreme antigen and antibody excess. He assumes that antibody is divalent and that the effective valency of antigen (N) is limited by the number of antibody molecules that can be packed around one antigen molecule. In the equivalence zone all valencies of antibody and all the effective valencies of antigen are satisfied in a complex in which each antibody molecule is combined with two antigen molecules. The molecular ratio of antibody to antigen in such a complex is $N/2$. Assuming also that antibody and antigen molecules are spherical he calculates N , and hence $N/2$, the ratio in the equivalence zone. Pauling also suggests various experiments to test his hypothesis.

How (29) has offered a simplification of the method by which Boyd & Hooker (30) calculated the ratio in the equivalence zone. Pauling pictures a complex of antigen molecules held together by divalent antibody molecules while Boyd & Hooker, and How conceive of single antigen molecules covered by monovalent antibody molecules. Nevertheless, the two types of calculation give approximately the same results, both agreeing fairly well with those found experimentally, because Boyd & Hooker, and How assume that antibody molecules consist of three spheres, each in contact with the antigen molecule (that is, that the antibody molecule is flattened and occupies more

space on the surface of the antigen molecule), while Pauling assumes that antibody molecules are spherical. Until the shape assumed by antibody molecules, when in combination with antigen, is known, these geometrical calculations can, at best, give limiting values. It might be hoped that the measurement of the actual thickness of antibody layers, attached to films of antigens, might decide this question of shape; but the answer given by such measurements is, at present, uncertain.

Kleczkowski's (31) work on the quantitative aspects of the serological reactions of plant viruses and a bacillus gives the ratios between antigen and antibody in precipitates shown in Table I.

TABLE I

Antigen	Particle weight of antigen	Range	Ratio of antibody N to antigen N	Number of molecules of antibody to one molecule of antigen
Tobacco mosaic virus (31).....	50×10^6	{ Antibody excess Equivalence	3.4 0.2	About 1,000 60
Bushy stunt virus (31).....	8×10^6	{ Antibody excess Equivalence	1.4 0.25 to 0.4	75 12 to 20
Pea nodule bacteria (31)	1×10^{11}	Equivalence	0.01	
Diphtheria toxin (7)	70×10^3	{ Antibody excess Equivalence Optimal flocculation point Antigen excess }	3.6	{ 8 4 to 1.5 2 1 to 0.5 }
Diphtheria toxin with β -globulin fraction (8).....	40×10^3	Optimal flocculation point	3.5	2
Diphtheria toxin with γ -globulin fraction (8).....	40×10^3	Optimal flocculation point	0.7	4
Egg albumin (horse anti-serum) (36)...	40×10^3	{ Antibody excess Equivalence	15.2 6.8	4 About 2

With bushy stunt virus as antigen the curves relating the amount of precipitate to amount of antigen added were similar to those ob-

tained when the antigen is a protein of lower molecular weight; but with tomato mosaic or aucuba mosaic as antigens the maximum precipitate was obtained far in the region of antigen excess. The ratios of antibody to antigen in the equivalence zone showed the diminution of this ratio with increasing size of antigen, as found by Boyd & Hooker (30).

Liu & Wu (35) used crystalline watermelon globulin as antigen and obtained the low ratio of 0.6 in the equivalence zone. It is probable that the particles of antigen were large, as it was insoluble at pH 7.7 in solutions of sodium chloride of a concentration lower than 2 per cent.

Pappenheimer and colleagues (7), having established the molecular weights of diphtheria antitoxin of horse serum and of diphtheria toxin, were able to calculate the molecular ratios, antibody to antigen, both in precipitates formed by toxin and antitoxin and in the soluble compounds formed when antibody or antigen are in excess (Table I). They concluded that the maximum number of antitoxin molecules that can combine with one toxin molecule is eight, while the maximum valence of antitoxin for toxin is definitely greater than one and at most two.

The reaction between diphtheria toxin and the antitoxin of horse serum has been regarded as exceptional, because precipitates are formed only within a narrow range of the ratio of antibody to antigen. The peculiarity cannot be ascribed to the antigen; for the reaction between diphtheria toxin and antitoxic sera formed in rabbits follows the normal course of reactions of protein antigens with rabbit antisera. Pappenheimer (36) has found that, in the reaction between crystalline egg albumin and horse antiserum also, no precipitate is formed when the ratio of antibody to antigen is twice or 0.66 times that at the equivalent point. Pappenheimer suggests that this type of reaction may be characteristic of the horse antisera to protein antigens, and inhibition in the zone of antibody excess has been attributed (7) to the asymmetric structure of the antibody molecule. However, the antisera to egg albumin, obtained in the early stages of immunization, contained nonflocculating, inhibiting antibody (36, 37). It is possible that inhibition in the zone of antibody excess may have been due to the continued presence of such antibody and comparable to the inhibition in the pro-zone found, for example, by Shibley (39) and Kleczkowski (32).

In the reaction between watermelon globulin and horse antiserum

(35) there was no evidence of inhibition by antibody excess. This appears to contradict Pappenheimer's suggestions, but it is possible that, in this case, the reaction was modified by a low degree of dispersal of the antigen and was more comparable with an agglutination than a precipitation.

Heidelberger & Kendall (37a) suggested that nonflocculating antibody may have had only one combining group per molecule and hence could not form a lattice; this suggestion was adopted by Pappenheimer (36) and Heidelberger and colleagues (37). It appears that the diphtheria antitoxin formed in the earliest stages of immunization may have half as many combining groups per molecule as that formed later (8). However, the first formed antitoxin flocculates more rapidly than that formed later, and appears to bind toxin more firmly.

Kleczkowski (32) has suggested another cause of failure of flocculation. He has confirmed the observation of Van der Scheer, Wyckoff & Clarke (34) that complexes between different fractions of serum proteins are formed when sera are heated. When whole antisera containing agglutinins of the O type or mixtures of partially purified agglutinin globulin with serum albumin are heated, such complexes are formed. These will not flocculate with antigen, but will combine with antigen and will inhibit flocculation by unheated serum. Agglutinins of the H type are not changed into inhibiting antibodies in this way when they are heated. Incidentally, a similar difference is found between O type and H type antigens when these are heated (33). It is possible that ability to flocculate with antigen depends, in other instances also, not on the number of combining groups, but on the nature of the rest of the antibody molecule.

There is evidence that the antibody molecules in a given serum are not uniform and that their character may change in the course of immunization. By the use of isotopic amino acids Schoenheimer, Heidelberger, Rittenberg & Rattner (40) have shown that, like other plasma proteins, formed and circulating antibodies are subject to rapid and extensive chemical changes. It is therefore possible that the character of antibodies may change after they have been formed and even that normal globulin may be converted into antibody.

The effect of temperature on the rate of reaction of antigen and antibody has been investigated by Hershey (43). The antigen used was coli-phage and the degree of neutralization of the phage was used as a measure of the extent of the reaction. The temperature coefficient of the reaction was 2 per 10° rise of temperature; it was esti-

mated that only 1 in 5×10^5 collisions resulted in reaction. Owing to the rapid increase in the proportion of effective collisions with rise of temperature it was concluded that the lowness of the proportion was not a matter of mutual orientation of antibody and antigen molecules, but that the reaction followed only if the colliding molecules shared a minimum kinetic energy of mutual translation.

Evidence of the rapidity of combination of antigen and antibody has been found in other investigations (37, 41, 42); Heidelberger and colleagues (37) found that complete combination takes place in less than twenty seconds.

The amount of precipitate formed by egg albumin with horse antiserum is unaffected by temperature (37); this system therefore resembles protein-antiprotein and polysaccharide-antipolysaccharide systems in rabbit sera, and not the pneumococcic polysaccharide-antipolysaccharide system in horse sera.

Using a sensitive calorimeter and taking advantage of the fact that horse antisera against protein antigens do not form precipitates in the region of antibody excess, Boyd and colleagues (41) have estimated the heat of combination of haemocyanin with antibody. The reaction was rapid, as 80 per cent of the heat was evolved within two minutes after mixing. The heat evolved was 3.3×10^6 cal. per mole of antigen and 4×10^4 cal. per mole of antibody. Previously Follensby & Hooker (42) had found that temperature had no influence on the equilibrium of the reaction between diphtheria toxin and antitoxin; the method that they employed was relatively crude.

Woelf (44) has published an interesting investigation of the quantitative aspects of inhibition by simple haptenes. He found that the inhibitory effect depends on the concentration of the hapten, not on the absolute amount present. Final results are approximately the same whether hapten is added when antigen and antibody are mixed or stirred up with the precipitate after it has been kept in the cold for twenty-four hours. Since nonflocculating antibody does not permanently inhibit the formation of a precipitate by egg albumin with rabbit antiserum (37), the mode of action of these two types of inhibitor must differ. Woelf suggests that the hapten removes part of the antibody completely from reaction and blocks some of the combining groups of other antibody molecules.

Gerlough and his colleagues (46) studied the relation between precipitin N, agglutinin N and protective power of rabbit antisera to pneumococci of Types I to V, VII, VIII, and XIV. Type specific

precipitin accounted for 86 per cent and type specific precipitin plus heterologous agglutinin for 93 per cent of the total agglutinin. After removal of the specific precipitin only 3.1 per cent of the protective power remained. They had also found (45) that the ratios of mouse protective units to precipitin nitrogen content of Types I to V, VII, and VIII rabbit antipneumococcic plasma, serum or concentrates ranged from about 900 units per mg. for Type II to 2,000 for Type VII. They felt that the greater part of the variation was due to the difficulty of determining the mouse protective units of the various types and of titrating the sera in terms of these. All fractions of globulin, obtained by precipitation with ammonium sulphate, were reasonably uniform with respect to the number of protective units per mg. of precipitin. Bjørneboe (47) had found that in rabbit antisera of Types I to VIII the protective power was proportional to agglutinin, if the immunization age was over five weeks. The lower ratios of young sera were ascribed a lower avidity of agglutinin for pneumococci. Bjørneboe (48) also found that the increase of protein in the sera of rabbits immunized against pneumococci was accounted for wholly by the agglutinin protein formed.

The quantitative experiments of Pappenheimer & Robinson (11) with diphtheria antitoxin prepared by the Parfentjev digestion process suggested that the antibody molecules are split by enzyme into an active and an inactive part. Similar work by Grabar (49), which has been confirmed by Treffers & Heidelberger (50), indicates that the molecules of antibody to pneumococcal polysaccharides are split by pepsin into approximately equal parts, one active, the other inactive. Pope's (51) studies of the effects of varying conditions, pH, time of digestion, etc., on the isolation of antibody by digestion with proteolytic enzymes have clarified this procedure and raised interesting points in connection with the structure and properties of antibodies. He finds that antibodies are readily split by digestion for a short time into an inactive portion, which is easily denatured by heat, and an active portion which is more resistant. Ordinary antitoxin contains 60,000-90,000 units and enzyme-treated antitoxin about 140,000 units per gram of protein (also found by Pappenheimer & Robinson).

The practical value of this method lies in the elimination of non-specific proteins. Various techniques depending on long digestion have proved less satisfactory (52). Since the digested antibody appears not to be antigenic, injection should not cause serum sickness nor anaphylactic shock. The method can be applied to tetanus antitoxin.

Coghill and colleagues (53) have used taka-diastase, which contains a mixture of enzymes, to reduce the antigenic effect of horse sera.

A new method by which antibody proteins can be labeled and identified in small amounts has been devised by Coons and colleagues (54). The β -anthryl carbamido derivatives of the proteins of anti-serum to Type III pneumococci were formed by treatment with β -anthryl isocyanate. The compounds fluoresced strongly in ultraviolet light. The degree of dilution in which the serum agglutinated Type III pneumococci was not altered by the treatment. The agglutinated pneumococci were fluorescent. If the treated Type III antiserum was mixed with Type II antiserum and Type II pneumococci were agglutinated by the mixture they were not fluorescent—another example of the freedom of specific precipitates from contamination with non-specific proteins.

Natural protein antigens.—Studies of the antigenic properties of antibody globulins were mentioned in the last review (1). Further quantitative investigations have been made by Treffers & Heidelberger (50). Rabbits were immunized with the floccules formed by the specific polysaccharide of Type II pneumococci and homologous horse antiserum. Using the rabbit antisera, so obtained, no antigenic differences were found between the horse antibody globulins against Type I and Type II pneumococci (free or combined with the homologous polysaccharide), against C-polysaccharide, and against *Haemophilus influenzae*. Horse antibody globulins against egg albumin and diphtheria toxin differed appreciably in their antigenic properties from these essentially antipolysaccharide antibodies. Digestion of pneumococcic antibody increased the number of sites for combining with specific polysaccharide and diminished the number of antigenic sites per unit weight of the antibody. The experiments show that an antibody globulin (G) has two distinct sets of specifically reacting sites: (A) antibody sites that combine with the homologous antigen, and (B) antigenic sites that combine with the new antibody formed against G when G itself is used for immunization.

A similar independence of two specific sites is found in beef catalase, as the enzymic activity is not impaired by combination with antibody [(55), compare urease (56)]. Antibody to beef catalase gives cross reactions with horse and dog catalase, but not with haemoglobin; nor is precipitation of catalase by anticatalase inhibited by haemoglobin or haematin. The serological specificity of catalase is, therefore, not determined by the iron-porphyrin group.

What is, possibly, a cruder example of this dissociation of specific groups is found in the thromboplastic lipoprotein prepared from lung (57). Reaction of this protein with antiserum is not impaired by removal of the lipid fraction, which destroys the coagulating power. The complex formed by the lipoprotein with antibody clots blood as rapidly as the free lipoprotein.

It has long been recognized that there is little difference between the lens proteins of not too distant species. Markin & Kyes (60) found that pigeons sensitized to ox lens protein are not sensitive to pigeon lens protein and are not desensitized by injection of pigeon lens protein, whereas dog and ox lens protein are practically equivalent. Pillemer & Ecker (61) consider that the methods of extraction used in previous work have been open to criticism. With avoidance of the risk of denaturation in the process of preparation they found little difference between sheep and swine lens protein, weak cross reactions between these two mammalian lens proteins and chicken lens protein and still weaker between chicken and fish lens proteins. There was little difference between sheep and swine proteins with respect to cysteine content and isoelectric point; while chicken protein differed appreciably in one direction and fish protein in the other. Oxidation of the sulphhydryl groups of the proteins weakened the reactions with antisera against the reduced proteins.

Hektoen & Welker (58) have confirmed the conclusion from previous work that Bence-Jones proteins fall into two groups that are immunologically distinct. Both types may be excreted by the same patient. They report that Medes found the nitrogen distribution similar in all but one of their preparations. The immunological behaviour of this one resembled that of other preparations from which it differed chemically. This suggests that the specificity of these proteins may be determined by a small part of the molecule. A more complete analysis of Bence-Jones protein has been published by Devine (59).

The cross reactions of certain virus proteins were studied by Chester (62) and by Bawden & Pirie (63). These studies have been continued by Kleczkowski, using quantitative methods (31). Aucuba mosaic virus behaved with tobacco mosaic virus antiserum in almost exactly the same way as did tobacco mosaic virus itself; but, with aucuba mosaic virus antiserum, tobacco mosaic virus did not precipitate all the antibody nor form as much precipitate as did aucuba mosaic virus. Knight & Stanley (64) have estimated some of the amino acids of viruses. Tobacco mosaic and aucuba mosaic viruses, which are

closely related immunologically, differ but slightly; while cucumber virus 4, which does not give cross reactions with tobacco and aucuba mosaic, contains much less tryptophane and much more histidine.

The basis of such cross reactions has been attacked further by Landsteiner & Van der Scheer (65). They studied the cross reactions of crystalline egg albumins of chicken, turkey, guinea fowl, duck, and goose. By absorption methods they showed that the cross reactions of these albumins could not be accounted for by the postulation of numerous determinant groups in the antigens and corresponding distinct antibodies in the antisera. For example, turkey egg albumin was added to antiserum to chicken egg albumin in sufficient amount to remove all antibody that would form a precipitate with turkey egg albumin. The formation of a precipitate by the exhausted serum with chicken egg albumin was inhibited by further addition of excess of turkey egg albumin. The cross reactions are ascribed to the similarity of determinant groups in the cross-reacting proteins. This point recurs below in connection with the experiments of Landsteiner & Van der Scheer with antigens containing peptides as determinant groups (78).

A thorough review of the proteins of acid-fast bacilli is given by Seibert (67). Later investigations into the relation of antigenicity to molecular weight have shown that, contrary to the earlier reports, fractions of tuberculin of molecular weight below 17,000 may be highly antigenic.

By means of the specific reaction with antiserum Landsteiner & Parker (66) have shown that serum proteins are made by chicken fibroblasts, grown *in vitro*, after repeated subculture.

Artificial protein antigens.—The reaction of egg albumin, denatured by hydrochloric acid at pH 1.5 to 2.3, with its antiserum has been studied by MacPherson & Heidelberger (68); it was similar to that of egg albumin with its antiserum. Cross reactions between egg albumin and denatured egg albumin were slight. Heidelberger, Davies & Treffers (69) phosphorylated egg albumin by treatment with phosphorous oxychloride. In the reaction of the phosphorylated protein with its antiserum there was no definite equivalence zone in which neither antigen nor antibody were present in the supernatant fluid. This antiserum formed little precipitate with egg albumin, but more with acid-denatured egg albumin. Some precipitate was formed with other artificial phosphorylated proteins, but not with casein.

The results of a number of recent investigations into the effects of substitution in the amino-groups of proteins on their immunological

behaviour may be summarized here (Table II). If the amino group of a protein P_1 (e.g., horse serum globulin) is treated to form P_1R , will (A) P_1R form a precipitate with antisera against P_1 , (B) will P_1 form a precipitate with antisera against P_1R , (C) will the product P_2R (made with another protein, e.g., egg albumin) form a precipitate with antisera to P_1R and (D) will the formation of a precipitate by P_2R and antisera to P_1R be inhibited by relatively simple compounds (AR) formed by treating amino groups of amino acids?

TABLE II

Amino groups treated with	A. Reaction of P_1R with antiserum to P_1	B. Reaction of P_1 with antiserum to P_1R	C. Reaction of P_2R with antiserum to P_1R	D. Inhibition by AR
Formaldehyde (70, 71)....	++	++	±	..
Phenylisocyanate (71, 72) ..	++	±	+	+
Dichlorodiethylsulphide ...	++	+	±	..
Dichlorodiethylsulphone (73).....	++	+	±	..
Benzylchloroformate (74)	±	+	++
Acetylsalicylazide (75)....	++	++

Cohn (76) also has found that guanidation of amino groups of serum albumin by treatment with methylisourea does not change the immunological properties of serum albumin greatly, although the albumin is rendered insoluble in distilled water. Kleczkowski (71), using quantitative methods in place of the traditional inspection and rows of plus signs, found (A) that proteins treated with formaldehyde or phenylisocyanate removed all the antibody from antisera to untreated proteins; (C) in the case of proteins treated with isocyanate, P_2R removed only a fraction of the antibody P_1R . These quantitative studies show that the reactions considered under (A) and (C) were not studied under strictly similar conditions, for the antisera to the unaltered proteins contained about ten times as much antibody nitrogen as did the antisera to the isocyanate-treated proteins. The cross reactions of weak sera cannot fairly be compared with those of strong sera. The specificity of proteins is little affected when the amino group is changed by treatment with formaldehyde; even when larger groups are attached at these sites the specificity may not be profoundly altered. The antigenic power, however, may be much reduced.

According to Kleczkowski (77), in the course of iodination of serum globulin the ability to react with and the ability to evoke antibodies to native serum globulin disappears when all tyrosine is substituted.

It is recognized that antisera against synthetic antigens contain qualitatively different antibodies, and it is possible that these may be antibodies against different parts of the determinant group. This possibility has been investigated further by Landsteiner & Van der Scheer (78) using polypeptides containing five amino acids as determinant groups in synthetic antigens. There was no evidence of the formation of antibodies specific against parts of the determinant group. Determinant groups with a terminal amide group in place of carboxyl were also used. There was little or no cross reaction between the two types of antigen. With the peptide-amide antigens the serological predominance of the carboxyl groups appears to have been suppressed by their conversion to amides.

Attempts have been made to make a practical use of the specific combination of antibodies with simple haptenes. Butler, Harington & Yuill (75) found that antisera to proteins combined with aspirin reduced the antipyretic action of aspirin, similarly to the neutralization of the action of thyroxine by antisera. These results are surprising, considering the small amounts of haptenes bound by antibodies *in vitro*. Hooker & Boyd (79) had attempted to prepare antimorpha sera, but had failed. They succeeded, however, in preparing antisera to antigens, formed by diazotizing mono-amino strychnine and coupling to proteins; the reaction of these antisera with strychnine-containing antigens was inhibited by strychnine and related compounds. But none of the sera contained more than 0.5 mg. per ml. of antibody nitrogen. None neutralized the lethal effect of strychnine for mice. In this, as in other attempts, Hooker & Boyd found it less easy to produce antibodies to basic than to acid determinant groups.

Goebel has continued to elucidate the relation of chemical structure to the specificity of pneumococcal polysaccharides by the use of synthetic antigens. The work with glucuronic acid, galacturonic acid and cellobiuronic acid was dealt with in the last review (1). Since then Goebel (80) has prepared a synthetic antigen containing gentiobiuronic acid, which differs from cellobiuronic acid in that the glucuronic acid is attached to the sixth in place of the fourth carbon atom of the glucose. This gentiobiuronic acid antigen formed precipitates with antisera to cellobiuronic and gentiobiose antigens; and antisera

against gentiobiuronic acid antigen formed precipitates with both cellobiuronic and gentiobiose antigens. The gentiobiuronic acid antigen formed a precipitate with antipneumococcic sera of Types III, V, and VIII; less with Type II serum. Rabbit antisera to the gentiobiuronic acid antigen agglutinated neither Type II nor Type III pneumococci, but protected mice against Type II infection. This protective power was removed by absorption with Type II cocci. As mice are also protected by antisera to glucuronic acid and cellobiuronic antigens, production of protective sera by gentiobiuronic acid antigens can be ascribed to the glucuronic acid part. Woolf (81) has published fuller details of the ingenious method by which he prepared a glucuronic acid antigen.

Polysaccharides.—Dingle & Fothergill (86) isolated from cultures of *Haemophilus influenzae*, grown on a casein hydrolysate medium, a polysaccharide containing only 0.3 per cent of nitrogen. It contained uronic acid and pentose and gave no biuret reaction. It formed precipitates with horse antiserum up to a dilution of 1 in 35×10^6 and with rabbit antiserum to 1 in 12×10^6 . Cross reactions were given with various antibacterial horse sera but not with rabbit sera. These cross reactions were probably not due to C substance. Hattie & Heidelberger (87) have shown by the quantitative agglutinin method that antibody to this polysaccharide is the chief antibody in anti-influenza sera, and may rise to amounts comparable with those found in antipneumococcic sera. When the polysaccharide was made from bacilli grown on agar it was heavily contaminated with agar.

Brown (88) prepared specific polysaccharides from pneumococci of Types I to XXXII. These formed precipitates with Type specific sera in dilutions up to 1 in 4 to 8×10^6 . Two (Types XXVIII and XXXII) contained 6 per cent of phosphorus, which was not all due to C substance or inorganic phosphate; twenty gave a reaction for amino sugars.

The whole structure of the specific polysaccharide of Type III pneumococcus seems to be settled, as Reeves & Goebel (82) have shown that the aldobionic acids are joined by a β -glucoside link between the glucose and third carbon atom of the next glucuronic acid.

A brief mention was made in the last review of studies of the relation between the specific polysaccharide of Type XIV pneumococcus and group A substance; fuller reports have since appeared (83). Erythrocytes of all four groups are agglutinated by horse Type XIV

antiserum in high dilution. Group A substance, prepared from commercial peptone, resembles Type XIV polysaccharide in optical rotation and in containing glucosamine, galactose, and acetic acid; but the group A substance probably contains a nitrogenous constituent not present in Type XIV polysaccharide (83b). As is usual, rabbit antisera are more specific than horse antisera. Group A substance forms no precipitate with rabbit Type XIV antiserum; with horse antiserum it forms a precipitate at 0°, but not at room temperature; and the precipitate formed at 0° redissolves on warming to 20° (83a). However combination appears to take place at the higher temperature, although no precipitate is formed; for the group A substance inhibits agglutination of human erythrocytes by the Type XIV antiserum at room temperature. About half the precipitable antibody is removed from the horse serum by precipitation with group A substance at 0°. Lysis of sheep's erythrocytes by anti-A rabbit serum was inhibited by group A substance in high dilution, but not by Type XIV polysaccharide.

Landsteiner & Harte (84) have described preparations of group A substances from pepsin and commercial mucin obtained from hogs' stomachs. These were as active as former preparations in inhibition of haemolysis but considerably less active in inhibiting isoagglutination. Amino acids were considered to be essential parts of the molecule. The same authors have made a short communication about the preparation of blood group substances from saliva (85).

The nature of the polysaccharide formed by a new organism, isolated from soil, has been studied by Kleczkowski & Wierzchowski (89). It is a polymannose of high molecular weight, composed of *l*-mannose units.

Recent studies of vegetable gums (90) give an indication of the types of structural differences which, apart from differences of component units, may lead to specific differences between polysaccharides. For example, the aldobionic acids of Type III pneumococcal polysaccharide lie in a single chain; those of gum acacia form side chains of a backbone formed of galactose units. The glucuronic acid units of Type III polysaccharide are attached by carbon atoms 1 and 4; in gum acacia a pentose chain is attached to carbon atom 4, and when this is removed the glucuronic acid is attached by its first carbon atom only.

Lipids and the syphilitic antigen.—Eagle & Hogan (126) compared the reactions of syphilitic serum with the Reiter strain of

spirochetes and with beef-heart lipid suspensions. Absorption of the serum with spirochetes removed all reactivity with beef-heart lipid suspensions while absorption with the lipid suspensions had no appreciable effect on the agglutinin titre for spirochetes. Immunization of rabbits with floccules, formed by beef-heart suspensions with syphilitic sera, raised the spirochete agglutinin titre of the rabbits' sera. The authors consider that the flocculating and complement-fixing reactions of syphilitic sera are cross reactions between antibodies to antigens present in spirochetes and antigens present in mammalian tissues. Concerning the nature of these antigens, Brown & Kolmer (127) found that the floccules formed in the Kahn reaction contain, besides protein, cholesterol and inactive diaminophosphatide, two active fractions that have the solubility characteristics of lecithin and cephalin. However, analysis of these two fractions shows that they are both contaminated to about the same extent by a nonnitrogenous phosphorus-containing substance. This may be the reacting substance.

Bier & Trapp (128) have shown that the reagin can be recovered from floccules, formed by the Eagle flocculating antigen with syphilitic serum, by extraction with strong salt solutions.

Complex bacterial antigens.—The concept that specificity may be determined by one part of a molecule or complex, while antigenicity, the power to evoke the formation of antibodies, may depend on another part, is derived mainly from studies of artificial antigens. But, from the discovery of the specific polysaccharides it was realized that this applied also to natural antigens; and complex antigens have been obtained from which could be isolated one fraction which carried all or part of the specificity, but was not antigenic. Recent studies have done something to clear up the parts played by the other fractions of these complexes. As mentioned in the last review (1) Morgan extracted an antigenic complex from *Shigella dysenteriae*. Morgan & Partridge (91) consider that this is a homogenous substance, ABC, composed of a phospholipin A, a polysaccharide B, and a "polypeptide-like" substance C. Immunization with ABC gives rise to antibodies similar to those produced by the intact organisms, together with a little precipitin against C. If A is removed, BC is still antigenic and gives rise to antibodies that both react with B and agglutinate the bacteria. A alone and AB are not antigenic. C on injection into a rabbit gives rise to precipitins against C or BC. A complex BC can be regenerated from isolated B and C. This com-

plex also is antigenic, giving rise to agglutinins against the bacteria and precipitins against B and C. Attempts to produce antibodies by injection of the polysaccharide, adsorbed on nonspecific substances, were unsuccessful. The polysaccharide, therefore, determines the specificity and the "polypeptide-like" component makes the complex antigenic. The latest work (92) suggests that this component is composed of a simple protein and a prosthetic group containing phosphorus, and that the special antigenic property is due to the prosthetic group. The only function that can be assigned to the phospholipin A is inhibition of the formation of antibodies to the "polypeptide."

Following up this observation Morgan & Partridge have made artificial complexes, containing the substance C and agar (93), gum acacia and cherry gum (95) and a polysaccharide with group A specificity prepared from commercial pepsin or mucin (94). Rabbits immunized with the agar complex produced sera specific against agar; these sera formed precipitates with the purified substance, kanten, prepared from agar up to a dilution of one in two million. Antisera against the gum acacia and cherry gum complexes formed precipitates with the homologous gums. Antisera against the group A complex agglutinated group A erythrocytes in dilution from one in three thousand to one in twelve thousand; but did not agglutinate group B erythrocytes above the normal level of dilution. The polypeptide-like substance appears to have a special power of forming antigenic complexes with polysaccharides.

Recent studies support the view that the complexes that have been isolated from the *Salmonella* group exist as such, in the bacteria. Freeman, Challinor & Wilson (96) compared the fractions isolated from *Bact. typhosum* Ty₂ and *Bact. typhimurium* by three different methods: (i) extraction with trichloroacetic acid, (ii) tryptic digestion and (iii) extraction with diethylene glycol. Walker (97) used a method of extraction with concentrated solutions of urea. The four methods gave similar products. Mackenzie, Pike & Swinney (98) also found that similar products were obtained by the first two methods. Freeman and his colleagues prepared the complex from bacteria grown in a synthetic liquid medium and showed that the fractions prepared from bacteria grown on agar may be highly contaminated with this polysaccharide. Freeman & Anderson (99) resolved the complex obtained by the tryptic digestion of *Bact. typhosum* Ty₂ into four fractions, a polysaccharide, an insoluble polypeptide, a soluble nitrogenous component, and a small lipid component. The

polysaccharide was strongly dextro-rotatory, contained 3 per cent of acetyl and consisted of *d*-mannose, *d*-galactose, *d*-glucose units; ketoses, pentoses, and uronic acids were absent. This polysaccharide appears to be the same as that isolated by Soru & Combiesco (100). It formed a precipitate with pure *Bact. typhosum* O-antiserum in a dilution of one in fifteen million, but not with Vi-antiserum and is to be regarded as the O-specific haptene of *Bact. typhosum*. The polypeptide is considered to render the complex antigenic. The amount of lipid component in this preparation was less than that found by Soru & Combiesco (100) and by Boivin & Mesrobian (101). The significance of this lipid and of the soluble nitrogenous component is uncertain.

Miles & Pirie (102) have approached the question of the nature of the antigenic complex of *Br. Melitensis* from a somewhat different angle. To obtain the complex with the minimum of disintegration they chose to extract the bacteria with chloroform water. They obtained a substance [PLAPS], from which a phospholipin PL and a protein-like substance S could be successively removed, leaving AP. Hydrolysis with weak hydrochloric acid split AP, liberating a phospholipin and a N-formylated amino-hydroxy compound A, which may be formylated amino-hexose. [PLAPS], which forms viscous opalescent solutions that show anisotropy of flow, is split on treatment with sodium dodecyl sulphate into smaller particles PLAPS, with molecular weights between 10^5 and 10^6 . AP has a molecular weight of about 10^6 , which can be reduced to between 1 and 2×10^5 . The amino compound, freed from formic acid, is fairly homogenous (103), with a particle weight of 3,300.

The antigenicity is in the order [PLAPS] > PLAPS > AP; the antigenicity of AP may be due to contamination with traces of PLAPS. [PLAPS] forms more precipitate with antisera than PLAPS and AP. A is not antigenic and forms no precipitate with antisera. These substances, when added in excess, inhibit the agglutination of *Br. Melitensis* by homologous antisera. The concentrations required for inhibition are in the order free amino compound > A > [PLAPS] > AP. The specificity therefore is carried by the amino compound, which behaves as a simple haptene.

Zittle (104) has investigated the constitution of two antigenic substances prepared from Type I haemolytic streptococci by mild methods—the type specific nucleoprotein M and the nucleoprotein N.P.A. which reacts more broadly. M substance contained only

ribose; its nucleic acids are therefore of the yeast type. N.P.A. contained both ribose and desoxyribose and, therefore, nucleic acids of both yeast and thymus type. Since the specificity of both M and N.P.A. was destroyed by protease and not by nuclease, it was concluded that the nucleic acid is not an essential part of either fraction.

Mudd & Lackmann (105) consider that the M substance prepared from a solution obtained by sonic disruption of streptococci is related to Lancefield's M and T, or both; the second N.P.A. had a broad reactivity, but evoked the production of type specific agglutinins. Again, the removal of nucleic acid did not cause any qualitative difference in the reactivity of the two fractions. A study of the properties of thin films of the two substances led Bateman and his colleagues (106) to the conclusion that the two fractions are chemically distinct. Also that the linkages between nucleic acid and protein are extremely labile, although other evidence led Heidelberger & Scherp (106a) to consider that much nucleic acid is bound more firmly than in dissociated salt linkages.

Lancefield's earlier work had shown that glossy variants of Group A haemolytic streptococci do not contain the M substance; yet they are agglutinated by type specific sera. Her recent work (107) shows that both matt and glossy streptococci contain another type specific antigen T, of which the chemistry is unknown. The antibody to this antigen contributes to the type specific agglutination. In the case of Type I, antibody to M does not agglutinate although it is present in sera and combines with M. As M antibody alone carries specific protection, the agglutinin titre of a serum is not a measure of its protective value. The strain C 203 is peculiar in that it contains M of Type III, and the T fraction of both Types III and I.

The distribution of these antigenic fractions in matt and glossy streptococci and in C 203 strain has been used by Henriksen & Heidelberger (108) in measuring the amounts of the antibodies in sera by the quantitative agglutinin technique. They found that the antibodies in sera may be mainly type or mainly group specific. The antibody to C polysaccharide is taken up in the type specific as well as in the group specific agglutination. However, obscure changes took place, on keeping, in both antigens and antisera, and reproducible results were less easy to obtain than with other systems.

Quantitative methods of study of antigen-antibody reactions were used by Scherp (109) as an indirect method of the investigation of antigens prepared from organisms. From the shape of the curve relat-

ing antibody precipitated to antigen added to a constant volume of antiserum he concluded that the polysaccharide prepared by himself and Rake (110) from meningococci is a mixture of precipitinogens, probably of a true type specific with a group specific polysaccharide.

Allergy to simple substances.—There have been important developments in connection with the production of allergy by simple substances, that may have an important bearing on human idiosyncrasies. Landsteiner & Chase (111) showed, first, that it is possible to produce sensitivity of the contact dermatitis type by intraperitoneal injection in place of applications of the incitant on or into the skin. Killed tubercle bacilli ground in paraffin oil were used as an adjuvant and picryl chloride as the incitant. Injections were made into the peritoneal cavities of seventy-six guinea pigs with care to avoid contaminating the skin. After three weeks sixty of these were sensitive to application of picryl chloride to the skin, most of them to a high degree. The correlation between capacity to sensitize guinea pigs and the ease with which substituents are replaceable [e.g., picryl chloride and anhydrides, Jacobs (114)] led to the supposition that the sensitization is due to conjugates formed in the body. However, attempts to sensitize with preformed conjugates by the usual technique were unsuccessful. If, however, repeated intraperitoneal injections of such conjugates were preceded by injection of dead tubercle bacilli, they sensitized guinea pigs to simple substances painted on the skin (112).

Second, although most simple substances that cause sensitivity have groups that are readily replaced, some more stable substances may cause sensitivity in man. Butesin picrate (a compound of one molecule of picric acid with two of the *n*-butyl ester of *p*-aminobenzoic acid) occasionally causes sensitivity in man when applied to burns. This suggested that sensitization is favoured by application to an injured area. Landsteiner & Di Somma (113) tried the application of picric acid to the skin of guinea pigs after cantharidin. Three weeks later reactions occurred when picric acid alone was painted on skin areas distant from the original site of application. Without the use of cantharidin, picric acid gave poor results. Picramic acid also was effective if used after cantharidin. Landsteiner & Di Somma suggest that sensitization may be due to reduction products that are more reactive than the original substance applied.

Complement.—Complement has been promoted from the status of a property of serum to that of a concrete substance that can be measured and isolated. In the first place Heidelberger (115a) has

shown that the absorption of complement leads to a considerable increase of the total nitrogen of an antigen-antibody precipitate. The fraction of complement that is bound in this way accounts for some 0.4 to 0.7 per cent of the total protein nitrogen of guinea pig serum. With Weil & Treffers (115b) he found that at limiting dilutions of antibody the amount of complement bound is about equal to the amount of antibody combined with antigen. Calculations based on the data obtained in these experiments and on the estimated sizes of the molecules involved show that only a small fraction of the surface of red blood corpuscles need be covered in order to produce haemolysis. Similar amounts of complement are bound in the zones of antibody and of antigen excess (116). In the second paper (115b) the mode of incorporation of complement into the structure of the antigen-antibody complex is discussed.

In the second place Pillemer, Ecker, Oncley & Cohn (117) have isolated mid- and end-piece by methods depending on solubility in dilute phosphate buffers. Both fractions are water-insoluble globulins with sedimentation constants approximately equal to those of normal serum globulin. The isoelectric point of mid-piece is at pH 5.2 to 5.4 and its mobility in an electric field at pH 7.7 is that of β -globulin. End-piece has an isoelectric point nearer neutrality (pH 6.3 to 6.4) but moves faster at pH 7.7, with the α -globulin fraction; it contains about 10 per cent of carbohydrate. Mid-piece makes up about 0.06 per cent and end-piece 0.18 per cent of the total protein of serum.

A number of points have been cleared by Pillemer, Ecker, and colleagues. All fractions of complement are precipitated by sodium sulphate and can be recovered with little loss from the precipitate (118). All fractions of yeast that adsorb third component are insoluble (119). A striking correlation is found between the adsorption of euglobulin-phosphorus and that of adsorption of mid-piece (120). It is suggested that mid-piece is closely associated with a phosphatide in the euglobulin fraction. This agrees with the location of mid-piece in the β -fraction. Free amino groups are required for inactivation of fourth component. Although all inactivators react with aldehyde groups, some aldehyde reagents such as hydroxylamine do not inactivate (121). Reducing agents do not inactivate fourth component (122); nor is its activity reduced by complete removal of calcium ions (123). The latter agrees with the conclusion of Gordon & Atkin (125). Pillemer & Ecker (123) consider that end-piece may be a calcium-carbohydrate-pseudoglobulin complex. End-piece is sensitive

to acids and mid-piece to alkalis (123). No definite relations exists between the inactivation of complement and the inhibition of coagulation by various agents (124).

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MICROBIOLOGY¹

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Most of the antibacterial agents available up to a few years ago belonged to the class of general protoplasmic poisons; their activity was usually explained in terms of their ability to denature the proteins of the microbial cell or to effect some other nonspecific toxic action, for instance, the oxidation of protoplasm constituents by the halogens and peroxides (1, 2). During the past decade there have been studied a number of entirely new types of antibacterial substances, synthesized by the methods of organic chemistry, or prepared from microbial, plant, and animal cells. The present review deals exclusively with the description of some of the chemical properties of these new substances, and with the attempts which have been made to explain their biological activity in terms of their effect upon the metabolic systems of the susceptible cells.

Sulfonamides and related compounds (2, 3, 4, 5).—It was in the form of azo dyes containing a sulfonamide group (in particular prontosil, the hydrochloride of 4'-sulfonamido-2,4-diaminoazobenzene) that the sulfonamides were first used as chemotherapeutic agents; these azo dyes, however, exhibit little if any antibacterial action *in vitro*. The fact that substitution in the second ring of these compounds does not destroy their antibacterial activity *in vivo*, whereas alteration in the sulfonamide ring has a profound effect, suggested that *p*-aminobenzenesulfonamide is the active part of the molecule. It is now generally—although not universally—believed, that the sulfonamide azo dyes owe their therapeutic activity to the fact that, in the tissues, they are reduced at the azo linkage, thus releasing the active *p*-aminobenzenesulfonamide *in vivo*: this substance, now commonly referred to as sulfanilamide, is indeed capable both *in vitro* and *in vivo* of inhibiting the growth of most bacterial species. It is well known that many derivatives of it have now been prepared, which exhibit selective activities against different groups of microorganisms. The present review will consider only the attempts which have been made to dis-

¹ This review is restricted to a discussion of bacteriostatic and bactericidal substances.

cover the chemical basis of the antibacterial activity of this class of compounds.

The addition of sulfonamides to bacteriological media can, under the proper conditions, inhibit the growth of many bacterial species, but, at 37° C. at least, it does not usually cause the death of the cells present in the inoculum (3, 6). The bactericidal effects of the drug, which have been observed at an incubation temperature of 40° C. (7, 8), may well be due to secondary effects. It is of great interest that the bacteriostatic effect does not take place immediately following the addition of the drug to the inoculated medium. Even the most susceptible bacterial species (group A streptococci for instance) may continue to multiply at a normal rate in the presence of an excess of the drug for several hours before cellular division eventually comes to a standstill. It is the existence of this lag period in the bacteriostatic effect which suggested that the real bacteriostatic agent is not sulfanilamide per se, but rather some product derived from it in the test tube or in the animal body. It was indeed established that oxidation products of the drug—in particular the hydroxylamino and nitroso derivatives—are extremely active bacteriostatic agents, more active than sulfanilamide itself (9, 10); the toxicity and lack of stability of these oxidation products make it difficult to appraise their effectiveness *in vivo*. The bacteriostatic effect of *p*-hydroxylaminobenzenesulfonamide does not exhibit the lag period which is so striking in the case of sulfanilamide, a fact compatible with the theory that the latter substance owes its activity to its oxidation products. As additional argument for the oxidation theory of the mode of action of sulfanilamide, it is pointed out that the intermediate products of oxidation of aromatic amino groups are powerful and reactive—although highly unstable—oxidizing substances. When reacting with oxygen, hydroxylaminobenzene compounds form strong oxidizing agents which exhibit antibacterial action providing the medium does not contain an excess of reducing substances capable of protecting the bacteria. The presence of the sulfonic group in the molecule of the drug tends to raise the oxidation-reduction potential and at the same time to increase solubility (11, 12). These hypothetical oxidation products must have a very transitory existence since sulfanilamide-containing media, in which bacteriostasis has occurred, and which therefore should contain the active oxidized agent, exhibit, when freed of bacteria and inoculated with new cells, a lag period in their bacteriostatic effect identical to that of new sulfanilamide media (13).

Several investigators have attempted to demonstrate the formation of these hypothetical oxidation products *in vitro* or during sulfanilamide therapy and have shown that the hydroxylamino derivative appears in the urine of treated patients (14). Evidence of the presence of oxidation products in blood has also been obtained by spectroscopic analysis (15). But even if it is accepted that the hydroxylamino derivative appears in the blood, it is not clear how it can act in this medium against bacteria because, when injected intravenously into dogs, it is completely converted to the reduced form in less than five minutes. Since the oxidized form can react so rapidly with blood pigments, only very small amounts of it can reach the bacteria. One would have to assume that in the course of therapy, the oxidation compound is formed on the surface or within the bacterial cell and acts immediately—an assumption difficult to prove or disprove (16, 17).

It is obvious that if the bacteriostatic activity of the sulfonamides is due to the formation of oxidized derivatives, bacteriostasis should fail to occur when oxidation is prevented, under anaerobic conditions for instance. A number of experiments with streptococci have dealt with this point, but with conflicting results. It would appear that the use of complex meat-infusion peptone media can hardly serve to decide this important question; it is possible that the hydrogen acceptors present in the medium can, when catalyzed by the organisms of the inoculum, oxidize the sulfonamides even when oxygen is absent from the surrounding atmosphere. It is desirable therefore that the effect of aerobic and anaerobic conditions on sulfanilamide bacteriostasis be again investigated in the synthetic media now available for most bacterial species. Observations of this nature have already been made with *Escherichia coli*; whereas this organism is not inhibited by sulfanilamide under aerobic conditions in a lactate or lactate-nitrate medium, bacteriostasis is observed in lactate-nitrate medium incubated anaerobically. Whether the nitrate acts as a hydrogen acceptor which permits the oxidative conversion of the drug into a bacteriostatic agent, or whether sulfanilamide per se inhibits the oxidation of lactate by nitrate, is still a moot question (8, 18, 19).

Many views have been offered concerning the phase of bacterial metabolism which is affected by the drug. In order to explain the chemotherapeutic effect, it was first assumed that the drug interferes with the production of capsules by pathogenic organisms, a view which has now been abandoned. Sulfanilamide, and especially its oxidation products (the hydroxyamides for instance) are known to inhibit the

enzyme catalase and therefore permit the accumulation of hydrogen peroxide (20). It has been claimed indeed that higher concentrations of this substance are found in cultures containing sulfanilamide. The hypothesis which considers the accumulation of peroxide as the immediate cause of bacteriostasis is of course compatible with a greater effectiveness of the drug under aerobic conditions, which some workers claim to have observed. This does not explain, however, the susceptibility *in vitro* of organisms which are known not to produce peroxide (3, 21), or the fact that the bacteriostatic action of sulfanilamide on streptococci is enhanced by the addition to the medium of blood, a rich source of catalase (22, 23). It may be pointed out at this time that the inhibitory effect of *p*-hydroxyaminobenzenesulfonamide is not limited to catalase, but affects other heme compounds; cytochrome-*c*, in particular, is irreversibly reduced by the substance (24). Sulfanilamide has also been shown to inhibit the oxidation of *p*-aminobenzoic acid by peroxidase (25).

Some early investigators assumed that sulfanilamide interferes in a more direct manner with the nutrition of the bacteria, either by combining with the substrate and rendering it unavailable to the cell, or by acting on the cell to prevent it from utilizing the substrate. Whereas serum does not affect the bacteriostatic effect of the drug, peptones and extracts of microbial cells and of animal tissues neutralize its effect. It was assumed that sulfanilamide prevents the utilization of certain foodstuffs (protein for instance) by inactivating some essential microbial enzyme (proteolytic enzyme for example). Peptones and tissue extracts containing substrates available to the bacterial cell without preparatory enzymatic action could offset this inhibition of enzyme (26). The problem of the nature of substances present in organic materials which inhibit the bacteriostatic action of sulfonamides has been made much clearer, and perhaps solved, by the discovery that *p*-aminobenzoic acid and related compounds are extremely active in this respect both *in vitro* and *in vivo* (27 to 35). This substance has been isolated from yeast in quantities sufficient to account for a large part of the antisulfanilamide activity of yeast extract (32, 33, 36, 37). Yeast extract also contains a sulfanilamide inhibitor, perhaps a peptide, which is not ether-soluble but from which *p*-aminobenzoic acid can be released by hydrolysis (37). This fact must be kept in mind in any study of the nature of inhibitors of sulfanilamide which are present in tissues and microbial cells, and which differ in solubility and other properties from *p*-aminobenzoic acid (38, 39, 40). Mention

can be made here of the fact that the development of sulfapyridine-fastness by pneumococci is accompanied by an increased production of sulfapyridine-inhibitor (39). It has been claimed, in the case of *Clostridium acetobutylicum*, that a mole of *p*-aminobenzoic acid is sufficient to inhibit the bacteriostatic effect of 23,000 moles of sulfanilamide (36). The significance of this observation is limited by the fact that the minimal concentrations of sulfonamide which are bacteriostatic vary greatly with the nature of the medium, and particularly with its content of sulfanilamide inhibitor (34, 39). It has also been found that coenzyme preparations from blood and yeast may inhibit the effect of sulfapyridine in preventing the growth of staphylococcus in synthetic medium; nicotinic acid shows no inhibitory action under the same conditions (41). It is unfortunate that synthetic coenzymes I and II are not available since coenzyme preparations obtained from natural sources may be contaminated with other compounds. This fact makes it difficult to reach a final conclusion as to the identity of the active substance (35). Methionine has been found to inhibit the bacteriostatic effect of the sulfonamides; arginine and lysine have been found to be only slightly inhibitory; and other amino acids have been found to be inactive in this respect (34, 35, 42, 43).

Shortly after the discovery of sulfanilamide inhibition by *p*-aminobenzoic acid, the latter substance was recognized to be an essential growth factor for certain microorganisms (32, 33, 36), and to be concerned perhaps in the metabolism of phenolic compounds (25). It appears that all substances related to *p*-aminobenzoic acid which can act as growth factors for *Clostridium acetobutylicum* also inhibit sulfanilamide action; conversely, derivatives of *p*-aminobenzoic acid which are inactive as growth factors are also inactive as inhibitors (31). These new findings have given weight to the theory that sulfanilamide exerts its bacteriostatic action by competing (on account of similarity in molecular structure) with *p*-aminobenzoic acid in some enzymatic reaction essential to growth (27, 44). A mathematical analysis of the growth rates of *Escherichia coli* growing in synthetic media containing sulfanilamide and *p*-aminobenzoic acid also gives evidence that the inhibition is of a competitive type (45). A more complete discussion of the theory is presented at the end of this review.

It has been repeatedly shown that the primary effect of sulfonamides on susceptible bacterial cells is bacteriostatic but not bactericidal. In fact, sulfonamides fail, under many conditions, to affect the oxygen uptake of cell suspensions. Streptococci and pneumococci

in nutrient media keep on respiring in the presence of the drugs; any apparent inhibition of respiration, as compared with the untreated controls, appears to be due only to failure of the cells to increase in numbers (46, 47, 48). There is some evidence, however, that sulfapyridine decreases the rate of oxidation of some of the three-carbon compounds by pneumococci, although it exerts no effect on the oxidation of glucose. A strain of *Pneumococcus* Type I which had become resistant to the drug, was also found to oxidize three-carbon compounds slower than did the susceptible parent strain (49). Sulfapyridine also inhibits the stimulation of respiration caused by the addition of nicotinamide to suspensions of dysentery bacilli; it seems that this inhibition is competitive since sulfapyridine must be added before the nicotinamide in order to obtain the effect (50). It will be noted that the few cases of inhibition of metabolism which have been recorded have been obtained with sulfapyridine. It would be of interest to study the effect of sulfanilamide on similar metabolic systems, to determine the possible role of the pyridine group in these reactions.

Before leaving the subject of the mode of action of sulfonamides, it may be of interest to state that these substances exert a number of biological effects, other than those already mentioned. Sulfanilamide strongly inhibits the enzyme carbonic anhydrase (although sulfathiazole and sulfapyridine do not) (51); it can stimulate plant growth and has a colchicine-like action, affecting the number of chromosomes in root tips and causing certain evolutionary changes (52, 53, 54); it may play a part in the phenomenon of bacterial dissociation (55, 56); it inhibits pellicle formation by *Bacillus subtilis*, without inhibiting diffuse growth (57); it inhibits the development of cultures of the algae *Schizococcus* by causing a reduction in the number of spores (58).

Although the sulfonamides have received so much attention because of their use in human therapy it must be remembered that the sulfonamide group is not essential to antibacterial activity. Mercaptans, disulfides, sulfinic acids, sulfonic acids, monosulfides, sulfoxides, and sulfones containing an aromatic nucleus have been reported to protect mice against pyogenic cocci. Organic compounds analogous to sulfones, but containing arsenic, phosphorus, or carbon instead of sulfur, *p*-nitrobenzoic acid and its derivatives, and a number of other substances of simple molecular structure have also been found to be endowed with antibacterial activity (2, 4, 5). It may be mentioned at this time that 2,3,5-triiodobenzoate has been found to affect the respiration and inhibit the growth of tubercle bacilli *in vitro* (59).

Surface active substances.—During the past two decades the requirements of industry have encouraged the synthesis of many synthetic compounds endowed with surface activity (detergents, wetting agents, etc.). These can be classified in three main groups depending upon the nature of the charge carried by the hydrophobic portion of the molecule,—anionic, cationic, or unionized compounds. Many of these substances have been found to exhibit remarkable bactericidal activity and to enhance the activity of other antiseptics (60, 61, 62, 63).

In general it can be stated that the anionic compounds (fatty acids, alkyl sulfates, etc.) affect only the Gram-positive bacterial species (63a). The cationic compounds (for instance alkyl pyridinium or quaternary ammonium halides) are often more active and are equally effective against Gram-positive and Gram-negative species. This difference in the bactericidal activity of the two classes of surface active compounds is reflected in their effect on the metabolism of bacterial cells. Dilutions of 1/3,000 to 1/30,000 of many cationic compounds immediately and irreversibly inhibit oxygen uptake and acid production of all species tested, whereas Gram-negative bacilli were found to be unaffected by anionic detergents (60).

Chaulmoogric and hydnocarpic acids are claimed to be effective in the treatment of leprosy, a fact which has suggested that other branched fatty acids might serve for the preparation of antiseptics active against acid-fast bacilli (64, 65). This view was encouraged by the knowledge that some of the most interesting components of the tubercle bacillus are also branched fatty acids (phthioic acid). A compound of chaulmoogra oil and β -glycerophosphoric acid, and a quaternary ammonium salt containing hydnocarpyl alcohol have indeed been found to be effective against the human tubercle bacillus *in vitro* (66).

The mechanism of the bactericidal effect of detergents has not been established. Antibacterial activity does not vary in proportion to the ability of the substance to depress surface tension (60, 62). Detergents are in general very effective protein denaturing agents (67), and can in particular dissociate conjugated proteins (68). They also act as peptizing agents and may thereby destroy the cell membrane. But any theory of their mode of action must explain: (a) the precise relationship between molecular structure and bactericidal action; (b) the extraordinary resistance of Gram-negative bacilli to anionic compounds; and (c) the marked protective effect of phospholipids against the antibacterial action of detergents (69).

Perhaps related to this problem, and also unexplained, is the possibility of sterilizing air by the spraying of certain glycols, many of which exhibit little if any antibacterial effect in solution (70, 71, 72).

Antibacterial agents of microbial origin.—The phenomenon of microbial antagonism has been repeatedly observed; a recent review lists 373 references which, directly or indirectly, bear on this subject (73). In the present discussion there will be considered only those cases of antagonism which have been shown to be due to the production of well-defined substances, other than the common metabolic products (organic acids, peroxides, etc.).

Under the name pyocyanase, extracts of *Pseudomonas pyocyanea* have been known for a long time to exert a marked antibacterial action which had been thought to be due to the presence of a bacteriolytic enzyme. It has now been shown that pyocyanase activity is in reality due to three substances which can be extracted with chloroform from old cultures of the organism: the pigment pyocyanine, α -oxyphenazine, and an oil which forms insoluble salts with barium, calcium, and heavy metals and which may be the substance described earlier under the name of pyocyanic acid (74, 75, 76). The antibacterial activity of the pigment pyocyanine may be due to its ability to inhibit the enzyme succino-dehydrogenase. Pyocyanine also interferes with the respiration of brain tissues and is a very toxic substance (77).

Aerobic sporulating bacilli and extracts of their cultures have often been found to inhibit the growth of other microbial species and have yielded a variety of active substances (73, 78, 79). From peptone cultures of *Bacillus brevis*, in particular, there have been separated a number of fractions all of which are endowed with antibacterial activity: a protein fraction, soluble in neutral aqueous buffers (80); and an alcohol-soluble, water-insoluble fraction, "tyrothricin," which appears to retain all the antibacterial activity of the original culture (79, 81, 82). Tyrothricin has yielded two crystalline components, gramicidin and tyrocidine, both of which are polypeptides, consisting in large part of amino acids with the *d*-configuration (82 to 87). Gramicidin which is slightly soluble in ether, does not possess any free basic or acid groups. It crystallizes in colorless platelets. Estimates of its molecular weight vary from 500 to 3,000. Its constituent amino acid residues include *l*-tryptophane, *d*-leucine, alanine, and an apparently unusual aminohydroxy acid. Tyrocidine contains free amino groups. Its hydrochloride is entirely insoluble in ether and can be crystallized from alcohol solutions as colorless needles. Its

molecular weight is a multiple of about 1260, perhaps 2534. The amino acid residues obtained from tyrocidine include tryptophane, tyrosine, alanine, phenylalanine, and a dicarboxylic amino acid (86, 88).

Both gramicidin and tyrocidine appear to be surface active compounds and their antibacterial action is inhibited by phospholipids. In spite of their common origin and similarity in chemical structure, they exhibit profound differences in biological activity. Tyrocidine behaves like a cationic detergent, bactericidal in buffer solutions for all bacterial species so far tested (with the exception of the tubercle bacillus), destroying immediately and irreversibly their metabolic activity (oxygen uptake, acid production, etc.). Gramicidin on the other hand is entirely inactive against Gram-negative bacilli, moderately active against meningococcus and gonococcus, selectively effective against Gram-positive cocci and bacilli. Under physiological conditions it does not destroy the respiration of susceptible cells; in the case of certain bacterial species at least, group D streptococci for instance, it is highly bacteriostatic but not at all bactericidal. For most tissue cells it is much less toxic than tyrocidine (with the exception of spermatozoa); it behaves more like a specific inhibitor of certain metabolic reactions than like a protoplasmic poison. This property may account for the fact that it retains much of its activity *in vivo* (79, 89).

Many strains of actinomyces possess the property of inhibiting the growth of certain bacterial species and of lysing living or dead bacterial cells. These different types of activity have been claimed to be due to a bacteriolytic enzyme called "actinomycetin" which is present in the extract of old cultures of a white *Actinomyces* (73, 90). It appears possible, however, that actinomycetin is not a single substance, but perhaps a mixture of toxic principles capable of killing certain bacterial species and of enzymes which digest the dead cells. From cultures of *Actinomyces antibioticus* there have been isolated two crystalline products: actinomycin A, which is soluble in ether and alcohol, but not in petroleum ether; and actinomycin B which is soluble in ether and petroleum ether, but only with difficulty in alcohol. The former is essentially bacteriostatic, the latter actively bactericidal (91).

Many molds also produce substances antagonistic to bacteria and other fungi. Preparations obtained from species of *Trichoderma*, *Glocladium*, and *Penicillium*, have been particularly well studied. In all three cases the active material is soluble in ether and chloroform

but sparingly soluble in water. Gliotoxin has been crystallized from benzene and alcohol in the form of silky white needles. Its composition corresponds to the formula $C_{14}H_{16}N_2S_2O_4$ (mol. wt. 347, as determined by depression of melting point). It is stable at acid reaction but very unstable on the alkaline side (73, 92, 93).

Culture filtrates of *Penicillium notatum* yield the antibacterial substance penicillin which is more active against the pyogenic cocci than against the Gram-negative bacilli (94, 95). Penicillin can be obtained in solution by extracting the culture medium with organic solvents, such as ether or amyl acetate, at pH 2.0. It is rapidly destroyed at acid reactions, but stable in the form of its neutral salts (96). It is also rapidly destroyed by a number of bacterial cultures. Its destruction by an enzyme extracted from *E. coli* does not involve any oxygen uptake (97). It does not seem that there is any relation between the susceptibility or resistance of bacterial species to penicillin and the absence or presence in these organisms of an enzyme capable of destroying the antibacterial substance.

Penicillin is primarily a bacteriostatic agent of very great activity, but bactericidal only to a small extent. It does not inhibit oxygen uptake by susceptible staphylococci (96). In its presence, susceptible bacteria keep on elongating but fail to divide, giving rise to giant and involution forms (98). The substance exhibits little toxicity for animal tissues, is not inhibited by serum or exudates, and retains its activity *in vivo* against localized and systemic infections (96).

One may mention at this time that there has been obtained from cultures of a certain staphylococcus a water-soluble, alcohol-insoluble substance supposed to be of enzymatic nature which lyses living corynebacteria (99). Very active antibacterial principles, active against both Gram-positive and Gram-negative bacteria, have also been recognized in culture filtrates of aspergilli (100, 101).

Antibacterial agents prepared from plant and animal tissues.—Only a few studies are on record of the antibacterial activity of plant tissues. Extracts of cabbage and turnip, of onion, and of barberry plants inhibit *Escherichia coli*, *Bacillus subtilis*, and other organisms (102, 103, 104). Chlorophyll derivatives and related compounds retard the growth of *Mycobacterium tuberculosis* (105). Anemonin extracted from *Ranunculus*, and the aromatic oils (in particular of *Allium sativum*) are also known to possess marked antiseptic properties (106, 107, 108).

On the other hand, attempts to explain natural immunity in higher

animals have led to the recognition of a vast variety of antibacterial substances in normal animal tissues. These agents, often heat stable, have been described under the name β -lysins, X-lysins, inhibins, mutins, bakterionoxins, etc. (109 to 118). Unfortunately they are too poorly defined chemically to warrant discussion in this review.

Lactenin is an agent present in cow milk which exhibits great activity against streptococci. Lactenin can be isolated from milk in the albumin fraction by precipitation with ammonium sulfate; it can be freed of salts and carbohydrates by dialysis. It is fairly stable between pH 4 and 10 and resistant to trypsin digestion (119, 120).

From many tissues and body fluids, it is possible to obtain an agent, lysozyme, capable of dissolving the cells of a number of microbial species (121, 124). Lysozyme is particularly abundant in egg white from which it can be conveniently prepared. Although reports of its crystallization (125) have never been confirmed, there is evidence that it has been obtained in a state of great purity by extracting desiccated egg white with acid alcohol, precipitating from this solution the active substance as the flavianate salt, and freeing it of flavianic acid with alcohol-ammonia mixtures (126). Very active preparations have also been obtained by fractional precipitation with alcohol (127). Lysozyme appears to be a basic polypeptide or a protein of low molecular weight and is very resistant to heat at acid reactions but is inactivated at alkaline reactions. It is considered to be an enzyme capable of hydrolysing certain mucopolysaccharides. In fact, one can isolate from susceptible bacterial cells a substrate—an acetylated aminopolysaccharide—which yields reducing sugars upon incubation with lysozyme. Similar results are obtained with the mucoid of egg white. It is likely, therefore, that the bacteriolytic activity of the agent depends upon its ability to destroy a polysaccharide structure of the susceptible bacteria (128, 129, 130). Morphological evidence of this destruction has been obtained from a microscopic study of the phenomenon of lysis. A photographic record of the process has revealed that the appearance of lysis does not result from a total destruction of the cell structure, but rather from a release of the cell contents into the medium (131).

In addition to lysozyme proper, tissues contain other antibacterial agents which differ in their selective activity against certain microorganisms and in their susceptibility to heat and other treatments (132).

General considerations.—As is well known, Ehrlich believed that

antiseptics and chemotherapeutic agents owe their antibacterial activity to the fact that they react with certain specific components of the susceptible cells. The name "receptors" was given to these hypothetical cellular structures for which the different antibacterial agents were supposed to exhibit specific affinities. Ehrlich's terminology is kept alive by a few workers who claim that bacteria possess different kinds of receptors; the α receptors are not essential to the viability of the cell, whereas alteration of the β receptors by antiseptics results in bactericidal effect (133, 134). In general, however, antiseptic action has in the past been analyzed in terms of nonspecific properties of the antibacterial substances such as their ability to cause protein denaturation or oxidation. On the contrary, recent investigators have again focused their attention on specific chemical reactions between inhibitors and cellular components, "the growth-inhibiting effect of antiseptics . . . in general might also be due to a specific poisoning of some essential reaction exerted through an interference with the catalyst or essential metabolite" (135).

The antiseptic activity of the mercuric ion had generally been ascribed to its ability to precipitate proteins. It is now pointed out that mercuric ion also possesses a great affinity for sulfhydryl groups and may thus interfere with cellular metabolism (135). It is a fact that the antibacterial effect of mercury can be specifically neutralized by the addition of sulfhydryl compounds to the system and can even be reversed by the same substances after prolonged exposure of the bacteria to the antiseptic; in the case of glutathione, the neutralization reaction takes place in proportion of one mercuric ion to two sulfhydryl groups. The hypothesis of the antisulfhydryl action of mercuric ion accounts for some of the facts incompatible with the view that mercurial germicides act by virtue of their ability to precipitate protein (for instance organic mercurials although less highly ionized than inorganic salts, are often more active against bacteria). It does not explain, however, the great antibacterial activity of sodium-ethyl-mercurithiosalicylate (merthiolate) a substance in which the mercury is already combined with a thiol group.

Most investigators of the germicidal activity of chlorine and iodine still emphasize the oxidizing power of these compounds (2). It has been established that undissociated phenolic compounds are far more germicidal than their phenolates (63, 136). It is also claimed that bacterial cells treated with phenol show a decrease of respiration before the killing effect can be observed (137). Certain

phenols and aliphatic alcohols inactivate succinic dehydrogenase (138). Hydroxy derivatives of phenothiazine react with heme pigments and in particular can reduce cytochrome-*c* irreversibly (139).

Little progress has been made concerning the nature of the antibacterial effect of dyes. It has been shown that the reaction of dye with bacteria is an exchange reaction, the dye replacing similarly charged ions already adsorbed on the cell (140, 141). Bacteria behave as electronegative colloids and fix electropositive but not electronegative dyes. The electropositive dyes bring about precipitation of the stained bacteria and slowly cause their death (142). A correlation has been established between physical and biological properties in the acridine series. The members most active against bacteria were found to be the most basic and the most hydrophilic. It has been pointed out, however, that biological activity may depend not so much on basicity as on the various types of amino groups which basicity indicates (143). Acridine dyes exhibit a profound depressing effect on the respiration of brain tissue (144). The enzyme succinodehydrogenase is readily inactivated by pyocyanine. No information is available to explain why, in general, basic dyes are much more effective against Gram-positive organisms than against Gram-negative species (61).

Attempts to explain antibacterial action in terms of specific inhibition of some essential metabolic steps have been greatly stimulated by the discovery that *p*-aminobenzoic acid can neutralize the bacteriostatic action of sulfonamides. It was assumed that *p*-aminobenzoic acid is an essential metabolite (27) (a fact now established experimentally) (32, 33, 36) and that the drug, by virtue of similarity in chemical structure, can compete with it for some enzyme essential to the bacterial cell. A number of other analogous types of inhibition have since been described. For instance, pyridine-3-sulfonic acid and its amide can inhibit bacterial growth by competing with nicotinamide (145). The aminosulfonic acid homologues of natural α -carboxyamino acids also inhibit bacterial growth. This inhibition of growth is decreased or removed by the addition to the medium of α -amino acids. Moreover, when staphylococci are made independent of most added aminocarboxylic acids by training, α -aminosulfonic acids are no longer inhibitory (146, 147). Inhibition of growth by *N*-(α,γ -dihydroxy- β,β' -dimethyl butyryl)-taurine can be reversed by the addition to the medium of pantothenic acid, the carboxyl homologue of the inhibitor, which is an essential growth factor (148, 149). Organisms which

synthesize their own pantothenic acid are not inhibited by the sulfonic acid compound. In all these cases it is likely that bacteriostasis is due to the blocking of reactions essential to growth by inhibitors which act on account of their structural similarity to the normal metabolite or growth factor involved. It is possible, therefore, that a rational approach to the production of new chemotherapeutic agents will consist in modifying the structure of essential metabolites so as to produce substances which can no longer exhibit any specific action in the cell economy, but are still able to block the enzymes concerned in the reaction (150, 151).

Another aspect, still very obscure, of selective relationship between the antibacterial substances and the bacterial cells, is offered by the differential susceptibility of Gram-positive and Gram-negative species to different agents (61). Basic dyes, anionic detergents, penicillin, actinomycin A, gramicidin, etc., are as a rule much more active against Gram-positive than against Gram-negative species. On the contrary, tellurites, azides, bichromates, etc., appear selective for many Gram-negative organisms (152 to 155). It is unknown whether this differential susceptibility is due to differences in cellular metabolism or to differences in permeability determined by the nature of the cell wall.

In the analysis of the mode of action of antibacterial agents, it may be profitable to keep in mind that susceptible bacterial species often give rise by "training" to variants endowed with great resistance to these agents (156). In some cases, drug resistance may be due to changes in metabolic behavior, for instance the ability to utilize a metabolic channel not blocked by the inhibitor, or the power to synthesize the aminocarboxylic acid homologue of an aminosulfonic acid used as inhibitor. It is also possible, on the other hand, that resistance may result from a change in cell permeability. It is known for instance that the failure of certain mutant strains of *Escherichia coli* to ferment lactose is not due to the absence of the enzyme lactase, but to the fact that the cell is not permeable to the disaccharide (157).

Oxygen uptake, acid production, and dehydrogenase activity have been the tests most commonly employed to determine the effect of antibacterial agents on the metabolism of the susceptible cells. It is of great interest that certain growth inhibitors (azide, dinitrophenol) not only do not abolish or decrease catabolic reactions but in fact greatly stimulate oxygen uptake by yeast, *E. coli*, and *Pseudomonas saccharophila*. It appears that these agents, by blocking some of the

synthetic reactions of the susceptible cells, allow complete oxidation of the substrates, and thus cause an apparent stimulation of metabolism (158 to 162). It will be recalled that in many cases the antibacterial agents considered in the present discussion (sulfonamides, gramicidin, actinomycin A, penicillin) can inhibit bacterial growth without abolishing oxygen uptake or destroying the viability of the susceptible cells. It may be justifiable to suggest, therefore, that our understanding of the mode of action of bacteriostatic and bactericidal agents has much to gain from a more accurate knowledge of the synthetic processes of the microbial cell.

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